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(54) Title: IMPROVED METHODS FOR ACHIEVING EXPRESSION FROM ALPHAVIRUS VECTORS

(57) Abstract: The present invention provides methods and reagents for enhancing alphavirus replication and gene expression in target cells by manipulating the PKR and/or IFN induced antiviral pathways present in the cell. The methods may also be practiced to expand alphavirus tropism to cells that are typically non-permissive, or only poorly so, for alphavirus replication and infection. The methods may be practiced in vitro, ex vivo or in vivo, for example, for the purposes of producing an alphavirus vector stock or a protein of interest in vitro, or for achieving immunogenic or therapeutic effects in a subject in vivo. Also provided are reagents (helper cells, nucleic acid constructs, helper sequences, alphavirus vectors, and the like) for practicing the methods of the invention.

Improved Methods for Achieving Expression from Alphavirus Vectors

Related Application Information

This application claims the benefit of U.S. provisional application serial numbers 60/367,776 and 60/367,893, both filed 25 March 2002, the disclosures of which are incorporated herein by reference in their entireties.

Statement of Federal Support

This present invention was made with government support under grant number RO1A122186-14 from the National Institutes of Health. The United States government has certain rights in this invention.

Field of the Invention

The present invention relates to improved methods of virus production and delivery, and reagents for carrying out the same. In particular, the present invention relates to improved methods of alphavirus production and delivery.

Background of the Invention

The alpha/beta interferon (IFNαβ) system provides a rapid, non-specific host response to counter a virus infection, both at the cellular and organismal level. The importance of IFNαβ for conferring viral resistance to the host is evidenced by the dramatically increased susceptibility to many viral infections of genetically-modified mice with the IFNAR1 subunit of the common IFNα and IFNβ receptor ablated (IFNAR1-/-; Byrnes et al. (2000) *J. Virol.* **74**:3905-3908, Chu et al. (1999) *Immunity* **11**:721-731, Fiette et al. (1995) *J. Exp. Med.* **181**:2069-2076, Garcia-Sastre et al. (1998) *J. Virol.* **72**:8550-8558, Grieder et al. (1999) *Virol.* **257**:106-118, Hwang et al. (1995)

Proc. Natl. Acad. Sci. U. S. A. **92**:11284-11288, Johnson, A. J. and J. T. Roehrig (1999) J. Virol. **73**:783-786, Mrkic et al. (1998) J. Virol. **72**:7420-7427, Muller et al. (1994) Science **264**:1918-1921, Rousseau et al. (1995) Journal of Interferon & Cytokine Research **15**:785-789, Ryman et al. (2000) J. Virol. **74**:3366-3378, Steinhoff et al. (1995) J. Virol. **69**:2153-2158, van den Broek et al. (1995) *J. Virol.* **69**:4792-4796, van den Broek et al. (1995) *Immunol. Rev.* **148**:5-18, White et al. (2001) *J. Virol.* **75**:3706-3718, Yeow et al. (1998) *J. Immunol.* **160**: 2932-2939). Sindbis virus, the prototypic member of the alphavirus genus, family *Togaviridae,* is one of the most IFNαβ-sensitive viruses known (Gomi et al. (1985) *Jpn. J. Cancer Res.* **76**:224-234, Overall et al. (1980) J. Infect. Dis. **142**:943, Petrillo-Peixoto et al. (1980) Intervirology **14**:16-20, Rebello et al. (1993) Acta Virol. **37**:223-231). It has have previously been demonstrated that a functional IFNαβ system, dependent upon signaling through the IFNAR receptor, is critical for protection of adult mice from fatal infection with Sindbis virus strain TR339 (Ryman et al. (2000) J. Virol. **74**:3366-3378). IFNαβ suppressed virus replication by an autocrine mechanism, but also specifically protected macrophages and dendritic cells (DC) from productive infection by the virus and prevented a fatal systemic inflammatory response syndrome (SIRS), characterized by the release of interleukin (IL)-12 p40, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and IL-6.

The IFNαβ system is extremely complex, exhibiting multiple, interconnecting levels of antiviral activity. The accumulation of partially double-stranded (ds) RNA viral replicative intermediates or secondary structure in the infected cell provides the primary proximal stimulus for a cascade of events (Jacobs, B. L. and J. O. Langland (1996) Virol. **219**:339-349). The presence of dsRNA in the host cell activates a number of constitutively expressed proteins with direct antiviral activity (Stark et al. (1998) Annu. Rev. Biochem. **67**:227-264), and potently induces expression from many cellular genes (dsRNA-stimulated genes [DSGs]), including the *ifna* and *ifnb* genes and other alarmone cytokines (Geiss et al. (2001) *J. Biol. Chem.* **276**:30178-30182). Activated antiviral and DSG-encoded proteins serve to suppress virus replication within the infected cell or rid the host of the

infected cell, and to trigger the production and release of IFNαβ. Exogenous IFNαβ signals through the IFNAR cell-surface receptor on infected and uninfected cells to upregulate expression of an array of IFNαβ-stimulated genes (ISGs; de Veer et al. (2001) *J Leukoc Biol* **69**:912-920, Der et al. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**:15623-15628). In the infected cell, autocrine IFNαβ signaling pathways greatly amplify the antiviral response, thereby further suppressing virus replication. Similarly, IFNαβ signaling "primes" uninfected cells by increasing levels of antiviral protein expression, which prevent or restrict initiation of virus replication. While priming appears to occur in most cell types, the autocrine effects of type I IFNs differ significantly between cell-types (Cella et al. (1999) J. Exp. Med. **189**:821-829, Schafer et al. (1998) J. Biol. Chem. **273**:2714-2720), perhaps dependent upon constitutive levels of IFN regulatory factors (IRFs). *In vivo*, low levels of IFNαβ production in the absence of virus infection may result in a constitutive priming effect in myeloid lineage cells, such as macrophages (Belardelli et al. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**:602-606). Considerable overlap exists in gene induction by dsRNA, virus infection, IFNαβ and other inflammatory cytokines demonstrating interconnections among disparate signaling pathways (Der et al. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**:15623-15628, Geiss et al. (2001) *J. Biol. Chem.* **276**:30178-30182, Vilcek and Sen (1996) *in* Fields Virology. Fields, D. M. Knipe, and P. M. Howley (eds.) Lippencott-Raven, pp. 375-399).

The two best-characterized dsRNA-triggered, IFNαβ-induced antiviral pathways are the coupled 2-5A synthetase/RNase L pathway, and the dsRNA-dependent protein kinase (PKR) pathway (reviewed in Stark et al. (1998) Annu. Rev. Biochem. **67**:227-264). The 2-5A synthetase/RNase L system is composed of the 2'-5' oligoadenylate synthetase (OAS) family of dsRNA-dependent enzymes and dormant, cytosolic RNase L. dsRNA-activated 2'-5' OAS synthesizes 2'-5' linked oligoadenylates (2-5A) that specifically bind and activate RNase L. Activated RNase L cleaves diverse RNA substrates, thus inhibiting cellular and viral protein synthesis. Constitutively expressed PKR is directly activated by binding of dsRNA and consequent autophosphorylation. A major substrate of PKR is the α-subunit of

the eukaryotic translation initiation factor 2 (eIF-2α). PKR-phosphorylated eIF-2α cannot be recycled, thereby greatly reducing the rate of translation initiation. Activated PKR phosphorylates IκB, resulting in NFκB release and the transcription of IFNβ and other ISGs, and plays a role in apoptosis of cells infected with certain viruses (e.g., Yeung et al. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**:11860-11865). In addition to these well-characterized pathways, antiviral activity has also been attributed to other proteins induced by IFNαβ and/or dsRNA including myxovirus resistance protein (Mx; 70), a tryptophan-degrading enzyme (Pfefferkorn (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**:908-912), adenosine deaminase (ADAR1; Patterson & Samuel (1995) *Mol.Cell. Biol.* **15**:5376-538853), ISG12 (Martensen et al. (2001) *Eur. J. Biochem.* **268**:5947-5954), ISG20 (Nguyen et al. (2001) *Biochemistry* **40**:7174-7179), ubiquitin-like protein ISG15 (Yuan & Krug (2001) *EMBO J.* **20**:362-371) and almost certainly other, as yet uncharacterized, factors. Even in the absence of the PKR, 2-5A/RNase L and Mx pathways (Iordanov et al. (2001) Mol. Cell. Biol. **21**:61-72, Zhou et al. (1999) *Virol.* **258**:435-440), alternative antiviral pathway(s) induced by IFNα treatment exhibit some residual antiviral activity against encephalomyocarditis virus (EMCV) infection both *in vitro* and *in vivo* (Zhou et al. (1999) *Virol.* **258**:435-440) and against dengue virus infection *in vitro* (Diamond et al. (2001) *Virology* **289**:297-311).

Earlier studies have demonstrated that Sindbis virus infection increases synthesis of IFNαβ *in vitro* and *in vivo* (Atkins, G. J. and C. L. Lancashire (1976) *J. Gen. Virol.* **30**:157-165, Klimstra et al. (1999) *J. Virol.* **73**:10387-10398, Rabinovich and Liu (1968) Experimental Medicine & Surgery **26**:117-23, Ryman et al. (2000) *J. Virol.* **74**:3366-3378, Svitlik and Marcus (1984) *J. Interferon Res.* **4**:585-602, Trgovcich et al. (1996) *Virol.* **224**:73-83). Recently, oligonucleotide array analysis of Sindbis-virus infected murine central nervous system tissue revealed the upregulation of many known ISGs (Johnston, C., W. Jiang, T. Chu, and B. Levine (2001) *J. Virol.* **75**:10431-10445). *In vitro* studies have suggested that both PKR (Saito (1989) *J. Interferon. Res.* **9**:23-34, Saito (1990) *Microbiol. Immunol.* **34**:859-870) and RNase L (De Benedetti et al. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**:658-662, Liu et al. (1983) *Scientia Sinica Series B, Chemical, Biological,*

Agricultural, Medical & Earth Sciences **26**:809-817, Schattner et al. (1982) J. Interferon Res. **2**:285-289) exert antiviral activity against Sindbis virus. However, these studies utilized immortalized fibroblasts as an *in vitro* model system; a cell-type with little relevance to alphavirus pathogenesis in nature.

Summary of the Invention

The present investigations have studied the effects of PKR/RNase L-dependent and alternative IFN induced-antiviral pathway(s) in eliciting an antiviral state in cells. The inventors have observed distinct effects of PKR/RNase L-dependent and alternative IFN induced-antiviral pathway(s) on initial infection of, and disseminated replication in, cells of the macrophage/dendritic cell lineage. Antiviral activity against Sindbis virus infection is only partially dependent on the combined PKR and 2-5A/RNase L pathways. Indeed, an alternative IFNαβ-induced pathway(s) can almost completely compensate for the absence of PKR and RNase L activity against Sindbis virus infection both *in vitro* and *in vivo*.

Accordingly, the present invention provides methods and reagents for enhancing alphavirus replication and gene expression in target cells by manipulating the PKR and/or IFN induced antiviral pathways present in the cell. The methods may also be practiced to expand alphavirus tropism to cells that are typically non-permissive, or only poorly so, for alphavirus replication and infection. The methods may be practiced *in vitro*, *ex vivo* or *in vivo*, for example, for the purposes of producing an alphavirus vector stock or a protein of interest *in vitro*, or for achieving immunogenic or therapeutic effects in a subject *in vivo*. Also provided are reagents (helper cells, nucleic acid constructs, helper sequences, alphavirus vectors, and the like) for practicing the methods of the invention.

The invention is discussed in more detail in the description of the invention set forth below.

Brief Description of the Drawings

Figure 1: Effects of antiviral pathways on the replication and dissemination of Sindbis virus TR339 *in vivo*. 5-7 week old 129 Sv/Ev

(black), TD (hatched), and IFNAR1-/- (white) mice were infected in the rear footpads with 100 PFU TR339 virus and sacrificed at the indicated time p.i. (Panel **A**) Virus titers in the DLN. (*) TD values significantly higher than 129 Sv/Ev or IFNAR1-/- ($p < 0.05$); (**) IFNAR1-/- values significantly higher than TD or 129 Sv/Ev ($p < 0.05$). (Panel **B**) Virus titers in DLN, serum, spleen and liver at 12 and 24 h p.i. Values represent the geometric mean virus titer (log_{10} PFU/ml or g) for three mice as determined by BHK cell plaque assay. Data are shown ± the standard deviation (SD).

Figure 2: Effects of antiviral pathways on SVRP-infected GFP-expressing cells in the DLN. 129 Sv/Ev (Panel **A**), IFNAR1-/- (Panel **B**), and TD (Panel **C**) mice were infected in the rear footpads with 10^3 GIU GFP-expressing SVRPs and DLNs were harvested 8 h p.i. 5 micron sections of DLN were photographed at constant shutter speed through a FITC filter on a Nikon TE300 fluorescence microscope (original magnification 200x). Arrows indicate GFP-positive cells.

Figure 3: Effects of antiviral pathways on TR339 replication in primary BMDC cultures. Primary BMDC were infected with TR339 virus and titers of progeny virions released into the supernatant were determined. Virus growth curves were generated in BMDC derived from 129 Sv/Ev (closed triangle), TD (closed circle) and IFNAR1-/- (closed square) mice infected at (Panel **A**) a low moi of 0.005 PFU/cell or (Panel **B**) a high moi of 5 PFU/cell. (*) TD values significantly higher than 129 Sv/Ev or IFNAR1-/- ($p < 0.05$); (**) IFNAR1-/- values significantly higher than TD ($p < 0.05$). **(C)** Virus growth curves in BMDC derived from C57BL/6 (open diamond), PKR-/- (open triangle) and RNase L-/- (open square) mice infected at high moi. (*) PKR-/- values significantly higher than RNase L-/- or C57BL/6 ($p < 0.05$). Values represent the geometric mean virus titer (log_{10} PFU/ml or g) for triplicate wells as determined by BHK cell plaque assay. Data are shown ± SD, where $n = 3$. Asterisk denotes statistical significance ($p \leq 0.05$).

Figure 4: Effects of induced IFNαβ on TR339 replication in primary BMDC cultures. BMDC were infected with TR339 virus in the presence or absence of 5000 NU anti-IFNαβ antibody and titers of progeny virions released into the supernatant were determined. Virus growth curves were generated in BMDC derived from 129 Sv/Ev with (open triangle) and without (closed triangle) anti-IFNαβ antibody, TD with (open circle) and without (closed circle) anti-IFNαβ antibody, and IFNAR1-/- (closed square) infected at high moi of 5 PFU/cell. (*) Addition of anti-IFNαβ antibody significantly increased virion production from TD BMDC ($p < 0.05$); (**) TD + anti-IFNαβ antibody significantly higher than IFNAR1-/- ($p < 0.05$). Values represent the geometric mean virus titer (log_{10} PFU/ml) for triplicate wells as determined by BHK cell plaque assay. Data are shown ± SD, where $n = 3$.

Figure 5: Effects of antiviral pathways on cpe in virus-infected BMDC. BMDC derived from IFNAR1-/- (Panel **A**), 129 Sv/Ev (Panel **B**), and TD (Panel **C**) mice were infected with TR339 virus at high moi (5 PFU/ml). In parallel wells, TD-derived BMDC were infected as above, but in the presence of 5000 NU anti-IFNαβ antibody (Panel **D**). The development of cpe in the infected BMDC cultures was followed by light microscopy. Cultures are shown 30 h p.i. (magnification 200x).

Figure 6: Effects of antiviral pathways on SVRP infection of BMDC *in vitro*: BMDC were infected at high moi with GFP-expressing SVRPs. GFP-positive cells in BMDC cultures derived from 129 Sv/Ev (Panel **A**), IFNAR1-/- (Panel **B**), and TD (Panel **C**) mice were photographed 8 h p.i. at constant shutter speed through a FITC filter (magnification 400x).

Figure 7: Effects of antiviral pathways on SVRP GFP expression in BMDC *in vitro*: BMDC were infected at high moi with GFP-expressing SVRPs and harvested for flow cytometric analyses. The mean fluorescence intensity of GFP expression from GFP-positive BMDC is shown. Mock-infected (white), 3 h p.i. (hatch), 6 h p.i. (black). Data represent the mean of $n = 2$ samples. These data were reproducible in three separate experiments.

Figure 8: Effects of antiviral pathways on induction of IFNαβ *in vivo* and *in vitro*. IFNαβ levels in (Panel **A**) serum samples virus-infected mice, and (Panel **B**) in the supernatant of virus-infected BMDC cultures were measured. Values represent the geometric mean IFNαβ titer (IU/ml) for triplicate samples as determined by biological assay. Data are shown as the mean of three samples.

Figure 9: Antiviral effect of IFNαβ or IFNγ priming in the presence or absence of PKR/RNase L. BMDC cultures derived from 129 Sv/Ev (Panels **A** and **B**), TD (Panels **C** and **D**) and IFNAR1-/- (Panels **E** and **F**) mice were pretreated for 12 h with IFNγ (Panels **A, C, E**) or IFNαβ (Panels **B, D, F**). Primed BMDC were then infected at high moi with TR339 virus and titers of progeny virions released into the supernatant were determined. The antiviral effects of a range of IFN pretreatment doses was tested; 1000 IU/ml IFNαβ or 1000 ED_{50}/ml IFNγ (open circle), 100 IU/ml IFNαβ or 100 ED_{50}/ml IFNγ (closed triangle), 10 IU/ml IFNαβ or 10 ED_{50}/ml IFNγ (open triangle), and untreated (closed circle). Values represent the geometric mean virus titer (log_{10} PFU/ml or g) for triplicate wells as determined by BHK cell plaque assay. Data are shown ± SD, where $n = 3$.

Figure 10: Antiviral effect of IFNαβ or IFNγ priming: BMDC were pretreated as described above with 100 IU/ml IFNαβ or IFNγ for 12 h prior to infection with GFP-expressing SVRPs. Cells were harvested for flow cytometric analyses 8 h p.i. The mean fluorescence intensity of GFP expression from the SVRP is shown. Untreated/mock-infected (white), untreated/infected (black); IFNαβ-treated/mock-infected (wide diagonal hatch), IFNαβ-treated/infected (narrow diagonal hatch); IFNγ-treated/mock-infected (wide vertical hatch), IFNγ-treated/infected (narrow vertical hatch). Data represent the mean of $n = 2$ samples. These data were reproducible in three separate experiments.

Detailed Description of the Preferred Embodiments

The present invention is based, in part, on the finding that both constitutively-expressed and interferon (IFN)-induced antiviral proteins can restrict alphavirus replication and tropism. Further, both constitutively-expressed and IFN-induced antiviral proteins may inhibit alphavirus gene expression. Moreover, the inventors have determined that there are at least two specific cellular pathways of IFN-mediated antiviral response that are effective against alphaviruses: (1) double-stranded RNA-dependent protein kinase (PKR), and (2) an alternative pathway that appears to be dependent upon signaling by the IFN receptor(s) (*e.g.*, IFN α/β and/or γ receptor).

Alphaviruses are sensitive to the antiviral effects of the IFN-mediated antiviral host response. Both *in vitro* and *in vivo*, induction and action of IFN α, β and γ may inhibit alphavirus particle production, intracellular virus replication and/or heterologous protein expression from the alphavirus 26S subgenomic promoter. In particular, cells of dendritic cell and macrophage lineages, important antigen presenting cells (APC), are very efficient at IFN response-mediated suppression of alphavirus expression. The present invention provides methods and reagents that may enhance (*e.g.*, increase) expression of heterologous sequences from recombinant alphavirus vectors *in vitro* and *in vivo* and improve alphavirus particle yields in *in vitro* viral production systems by inhibition of constitutive and/or IFN-mediated antiviral response.

In a simplified model, the effects of the IFN-mediated antiviral pathways may be observed at two levels. First, low level constitutively expressed antiviral proteins, particularly PKR and 2'-5' oligoadenylate synthetase, are activated by the accumulation of partially dsRNA viral replicative intermediates when the cell is infected, and the production and release of IFNαβ is triggered. The released IFNαβ is believed to act on the infected cell in an autocrine fashion via the IFN α/β receptor. On a second level, the release of IFNαβ from infected cells will "prime" surrounding and distal uninfected cells by signaling through the IFNαβ receptor, and confer an antiviral state upon them by upregulating the expression of antiviral proteins. The present invention is based, in part, on the inventors' discovery as to how

IFN-mediated pathways may be modulated to enhance alphavirus replication and transgene expression in initially infected cells and, in particular embodiments, this effect on initially infected cells is achieved while maintaining the antiviral defenses of surrounding and distal uninfected cells.

PKR is present at basal constitutive levels in cells, such as macrophages and dendritic cells. Interferon signaling induces cellular PKR activity. The present invention provides methods of inhibiting PKR (constitutive and/or interferon-induced) and/or IFN signaling pathways which can enhance alphavirus replication, new particle production and/or transgene expression *in vitro* and/or *in vivo*.

The present invention can further be practiced to expand viral tropism to cells of interest. PCT publication WO 97/24447 to Song et al. describes manipulation of viruses (*e.g.*, alphaviruses) to confer a dendritic cell targeting element thereto. It is commonly believed in the art that macrophages and dendritic cells are not permissive for infection by at least some alphaviruses (*e.g.*, Sindbis). While not wishing to be bound by any particular theory of the invention, the present inventors have discovered that macrophages and dendritic cells are, in fact, permissive for infection by Sindbis virus. However, constitutively-expressed and interferon-induced cellular antiviral mechanisms (*e.g.*, PKR and other interferon-stimulated defenses) effectively limit Sindbis replication in these cells. By inhibiting one or both of these mechanisms, the present inventors have found that alphaviruses (including Sindbis) may infect and replicate in macrophages/dendritic cells. Moreover, higher levels of alphavirus particle production and/or transgene expression may be achieved in macrophages, dendritic cells, and other cell types by inhibition of PKR and/or interferon-induced antiviral responses.

Except as otherwise indicated, standard methods known to those skilled in the art may be used for the construction and use of recombinant nucleotide sequences, vectors, helper constructs, transformed host cells, selectable markers, alphavirus vectors, viral infection of cells, production of attenuated viruses, and the like. Such techniques are known to those skilled in the art. *See, e.g.*, SAMBROOK *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. AUSUBEL *et al.*

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

I. Definitions.

The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

As used herein, the term "polypeptide" encompasses both peptides and proteins.

The term "alphavirus" has its conventional meaning in the art, and includes Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Encephalitis virus (WEE), Sindbis virus, South African Arbovirus No. 86 (S.A.AR86), Girdwood S.A. virus, Ockelbo virus, Semliki Forest virus, Middelburg virus, Chikungunya virus, O'Nyong-Nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzlagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, Buggy Creek virus, and any other virus classified by the International Committee on Taxonomy of Viruses (ICTV) as an alphavirus.

Preferred alphaviruses for use in the present invention are Sindbis virus strains (*e.g.*, TR339), VEE (having a mutation at nucleotide 3 of the genomic RNA following the methylated cap), S.A.AR86 virus, Girdwood S.A. virus, and Ockelbo virus, and chimeric viruses thereof. The complete genomic sequences, as well as the sequences of the various structural and non-structural proteins are known in the art for numerous alphaviruses and include: Sindbis virus genomic sequence (GenBank Accession Nos. J02363, NCBI Accession No. NC_001547), S.A.AR86 genomic sequence (GenBank Accession No. U38305), VEE genomic sequence (GenBank Accession No. L04653, NCBI Accession No. NC_001449), Girdwood S.A genomic sequence (GenBank Accession No. U38304), Semliki Forest virus genomic sequence (GenBank

Accession No. X04129, NCBI Accession No. NC_003215), and the TR339 genomic sequence (Klimstra et al., (1988) *J. Virol.* **72**:7357; McKnight et al.,(1996) *J. Virol.* **70**:1981).

Also preferred are alphaviruses that are particularly sensitive to the antiviral state induced by IFN and/or PKR.

An "Old World alphavirus" is a virus that is primarily distributed throughout the Old World. Alternately stated, an Old World alphavirus is a virus that is primarily distributed throughout Africa, Asia, Australia and New Zealand, or Europe. Exemplary Old World viruses include SF group alphaviruses and SIN group alphaviruses. SF group alphaviruses include Semliki Forest virus, Middelburg virus, Chikungunya virus, O'Nyong-Nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, and Una virus. SIN group alphaviruses include Sindbis virus, South African Arbovirus No. 86, Ockelbo virus, Girdwood S.A. virus, Aura virus, Whataroa virus, Babanki virus, and Kyzylagach virus.

The phrase "alphavirus structural protein(s)" as used herein refers to one or more of the proteins that participate in production of a functional alphavirus particle that encapsidates the alphavirus genomic RNA. The alphavirus structural proteins include the capsid protein, E1 glycoprotein, E2 glycoprotein, E3 protein and 6K protein. The alphavirus particle comprises the alphavirus structural proteins assembled to form an enveloped nucleocapsid structure. As known in the art, alphavirus structural subunits consisting of a single viral protein, capsid, associate with themselves and with the RNA genome to form the icosahedral nucleocapsid, which is then surrounded by a lipid envelope covered with a regular array of transmembranal protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2 (*See* Paredes et al., (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9095-99; Paredes et al., (1993) *Virology* **187**, 324-32; Pedersen et al., (1974) *J. Virol.* **14**:40).

The phrase "sequences encoding the alphavirus structural proteins sufficient for producing an alphavirus particle" as used herein refers to a nucleic acid sequence(s) (*e.g.*, DNA or RNA) that encodes at least those alphavirus structural proteins, or portions thereof, that are required to produce a functional alphavirus virion (typically, containing at least capsid, E1 and E2) that can

package alphavirus genomic RNA. The sequence(s) may be RNA sequences; alternatively, the RNA coding sequences for the alphavirus structural proteins may be expressed from DNA. As a further alternative, the nucleic acid sequence(s) may encode a functional portion of the alphavirus structural protein(s) or a mutated form (*e.g.*, attenuated) thereof. The alphavirus structural proteins may be encoded by a single nucleic acid molecule or by two or more (*e.g.*, three) nucleic acid molecules. The nucleic acid sequence(s) may be carried by an alphavirus genomic RNA or a separate helper sequence. Alternatively, they may be integrated into the cellular DNA (*e.g.*, in a packaging cell) or carried by a cellular episome (*e.g.*, EBV based episomes).

An "alphavirus genomic RNA" indicates the alphavirus RNA transcript, including recombinant and other genetically modified forms. The wild-type alphavirus genome is a single-stranded, messenger-sense RNA, modified at the 5'-end with a methylated cap, and at the 3'-end with a variable-length poly (A) tract. The viral genome is divided into two regions: the first encodes the nonstructural or replicase proteins (nsP1-nsP4) and the second encodes the viral structural proteins (Strauss and Strauss, *Microbiological Rev.* (1994) **58**:491-562). As used herein, the term "alphavirus genomic RNA" encompasses recombinant alphavirus genomes (*e.g.*, containing a heterologous nucleotide sequence), viral genomes containing one or more attenuating mutations, deletions, insertions, and/or otherwise modified viral genomes. For example, the "alphavirus genomic RNA" may be modified to form a double-promoter molecule or a replicon (each as described below).

A "chimeric" alphavirus as used herein comprises alphavirus structural proteins from one (or more) alphaviruses and a genomic RNA from another alphavirus. In embodiments of the invention, the chimeric alphavirus comprises a Sindbis genomic RNA and alphavirus structural proteins from another alphavirus (*e.g.*, VEE, GirdwoodS.A., Ockelbo, and the like). In other embodiments of the invention, the chimeric alphavirus comprises Sindbis alphavirus structural proteins and a genomic RNA from another alphavirus (*e.g.*, VEE, GirdwoodS.A., Ockelbo, and the like). In particular embodiments, the alphavirus structural proteins comprise structural proteins from two or more alphaviruses.

As used herein, the term "alphavirus vector" or "alphavirus gene delivery vector" refers to an alphavirus particle that functions as a delivery vehicle, and which comprises the alphavirus genomic RNA packaged within an alphavirus enveloped nucleocapsid. Alternatively, in some contexts, the term "vector" may be used to refer to the alphavirus genomic RNA, which may also be used to for the purposes of gene delivery, *e.g.*, by electroporation, lipofection, and the like.

An "infectious" alphavirus particle is one that can introduce the alphavirus genomic RNA into a permissive cell, typically by viral transduction. Upon introduction into the target cell, the genomic RNA serves as a template for RNA transcription (*i.e.*, gene expression). The "infectious" alphavirus particle may be "replication-competent" (*i.e.*, can transcribe and replicate the alphavirus genomic RNA) and "propagation-competent" (*i.e.*, results in a productive infection in which new alphavirus particles are produced). In embodiments of the invention, the "infectious" alphavirus particle is a replicon particle (as described below) that introduces the genomic RNA (*i.e.*, replicon) into a host cell, is "replication-competent" to replicate the genomic RNA, but is "propagation-defective" or "propagation-incompetent" in that it is unable to produce new alphavirus particles in the absence of helper sequences that complement the deletions or other mutations in the replicon (*i.e.*, provide the structural proteins that are not provided by the replicon).

As used herein, an "isolated" nucleic acid (*e.g.*, an "isolated DNA" or an "isolated alphavirus genomic RNA" or "isolated alphavirus genomic RNA transcript") means a nucleic acid separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the nucleic acid.

Likewise, an "isolated" polypeptide means a polypeptide that is separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide.

As used herein, the terms "deleted" or "deletion" mean either total deletion of the specified segment or the deletion of a sufficient portion of the specified segment to render the segment inoperative or nonfunctional, in accordance with standard usage (*see, e.g.,* United States Patent No. 4,650,764 to Temin et al.).

As used herein, "a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity" or "signaling activity" is intended broadly and encompasses any compound or agent including organic and inorganic compounds, and including polypeptides and nucleic acids. The substance may be provided in a precursor form to the cell, which may then convert the compound to the active form. Likewise, the substance itself may be provided to the cell (*e.g.*, a polypeptide); alternatively, a nucleic acid sequence encoding the substance (which may be a polypeptide, an antisense sequence, an interfering RNA, or other non-translated RNA) may be provided to the cell. The substance "inhibits" (*e.g.*, reduces or decreases) "cellular PKR pathway activity" or "signaling activity" such that at least one indicator of PKR activation is diminished. By "inhibit" or "inhibits" it is not necessary that all detectable PKR or PKR- induced activity is blocked, although this is the case in particular embodiments. Inhibition of cellular PKR pathway activity or signaling activity may be reduced by at least about 25%, 40%, 50%, 60%, 75%, 80%, 90%, 95%, or 99% or even more as compared with a suitable control cell (*e.g.*, a cell that is not exposed to the PKR pathway inhibitory substance). Cellular responsiveness to PKR may be achieved by inhibiting or interfering with any point(s) in the PKR signaling pathway, *e.g.*, interfering with expression of the PKR protein, activation of the PKR protein (*e.g.*, autophosphorylation), the function of the PKR protein itself, or a downstream signaling molecule (*e.g.*, NF_KB). The substance may inhibit constitutive (*e.g.*, basal) and/or interferon-induced PKR activity in the cell, preferably both. PKR pathway activity or PKR activity may be assayed by any technique known in the art, including Western blots of protein concentrations, antibody-based assays, or bioassays measuring biological responses associated with activation of the PKR pathway (*e.g.*, PKR autophosphorylation or e1F-2α phosphorylation assays). Likewise, PKR protein can be detected by standard protein detection methods.

Substances that inhibit cellular PKR pathway activity are known in the art and include, but are not limited to, 2-aminopurine, adenovirus VAI RNA, influenza A Ns1 protein, vaccinia E3L protein, Herpes simplex virus ICP34.5 protein, p58ipk protein, an autologous dominant negative PKR inhibitor (*e.g.*, having a Lys → Arg substitution at position 296; Terenzi et al., (1999) *Nucleic Acids Research* **27**:4369), an antibody (*e.g.*, that binds to PKR), an antisense nucleic acid, or an interfering RNA.

A "cell having a reduced double-stranded RNA –dependent (PKR) pathway activity" or "signaling activity" as used herein has a lessened constitutive and/or induced (*e.g.*, IFN-induced) PKR pathway activity as compared with a suitable control cell. The cell has a similar phenotype to a cell in which the PKR pathway has been inhibited with a PKR pathway inhibitory substance, as described above, except that it is a constitutive or inducible property of the cell. The reduced responsiveness may be a property of the cell that may exist in the native state. Alternatively, the cell may be genetically modified to reduce PKR pathway activity, for example, by modifying the cell by gene disruption of the gene(s) encoding the PKR protein or a gene encoding a signaling molecule within the PKR pathway, by modifying the cell to stably express an antisense RNA or inhibitory RNA to a gene encoding the PKR protein or a polypeptide within the PKR pathway, or by otherwise modifying the cell so that it has the desired phenotype. The nature of the defect in the PKR pathway need not be identified or attributable to a single alteration in the cell. For example, mutagenesis or cell selection techniques, as known by those skilled in the art may be employed to produce a cell having the desired characteristics. The level of PKR pathway activity is preferably substantially reduced, *e.g.*, by at least about 50%, 60%, 75%, 85%, 90%, 95%, 99% or more as compared with a suitable control.

In particular embodiments of the invention, the reduction in PKR pathway activity is attributable, at least in part, to a reduction in PKR activity (due to reduced PKR protein and/or activity of the protein). The level of PKR activity is preferably substantially reduced, *e.g.*, by at least about 50%, 60%, 75%, 85%, 90%, 95%, 99% or more as compared with a suitable control. Alternatively, and

preferably, the cell has been modified such that it "lacks detectable PKR activity" as determined by any suitable assay.

As used herein, "a substance that inhibits cellular responsive to interferon" is intended broadly and encompasses any compound or agent including organic and inorganic compounds, and including polypeptides and nucleic acids. The substance may be provided in a precursor form to the cell, which may then convert the compound to the active form. The substance itself may be provided to the cell (*e.g.*, a polypeptide); alternatively, a nucleic acid sequence encoding the substance (which may be a polypeptide, an antisense sequence, an interfering RNA, or other non-translated RNA) may be provided to the cell. The substance "inhibits" (*e.g.*, reduces or decreases) "cellular responsiveness to interferon" such that at least one indicator of IFN activation is diminished (*e.g.*, antiviral activity). By "inhibit" or "inhibits" it is not necessary that all detectable IFN or IFN- induced activity is blocked, although this is the case in particular embodiments. Inhibition of cellular responsiveness to IFN may be reduced by at least about 25%, 40%, 50%, 60%, 75%, 80%, 90%, 95%, or 99% or even more as compared with a suitable control cell (*e.g.*, a cell that is not exposed to the IFN inhibitory substance). Cellular responsiveness to IFN may be achieved by inhibiting or interfering with any point(s) in the IFN signaling pathway, *e.g.*, interfering with expression of one or more of the IFN receptors or a subunit thereof; blocking binding to or activation of the IFN receptor(s), or inhibiting a downstream signaling molecule in the interferon pathway. Cellular responsiveness to interferon may be determined by any suitable method known in the art for detecting interferon induced responses in a target cell, such as bioassays, detection of RNA or proteins known to be induced by interferon, and the like. For example, the level or activity of PKR, Rnase L, and/or 2'-5' oligoadenylate synthetase may be measured. Further, reductions in the IFN-induced antiviral state can be used as an indicium of IFN responsiveness, *e.g.*, by evaluating IFN-induced protection of cells (*e.g.*, L929 murine fibrosarcoma cell line) from EMCV (encephalomyelitis virus) induced cytopathic effects.

There are at least two IFN receptors, the α/β receptor (called the IFNAR receptor interchangeably herein) which binds to and is activated by IFN α and β and the IFNγ receptor, which mediates signaling by IFN-γ. These receptors

activate multiple pathways within the cell, including PKR, RNase L, and Mx-1 pathways. In embodiments of the invention, the substance interferes with multiple (*i.e.*, two or more) IFN signaling pathways so that overall IFN responsiveness of the cell is blunted, typically by inhibiting the IFN receptor itself (*e.g.*, by inhibiting expression of the receptor protein or one of the subunits thereof, assembly of the subunits and/or insertion into the membrane, ligand binding to the receptor, or activation of the receptor upon ligand binding). A "substance that inhibits cellular responsiveness to interferon" will generally inhibit multiple IFN pathways (*e.g.*, by interfering with the IFN receptor), and excludes substances that act only by inhibiting PKR pathway activity, as described above. In embodiments of the invention, the substance interferes with IFNAR receptor expression, assembly, ligand binding, and/or activation. Alternatively, or additionally, the substance(s) may interfere with the IFN-γ receptor expression, assembly, ligand binding, and/or activation.

Substances that inhibit cellular responsiveness to IFN are known in the art and include, but are not limited to, Ebola VP35 protein, bunyavirus NS protein, Sendai C protein, simian virus 5 V protein, vaccinia virus VH1 protein, an antibody (*e.g.*, that binds to IFN or an IFN receptor), an interferon receptor antagonist, an antisense nucleic acid, or an interfering RNA.

A "cell having a reduced responsiveness to interferon" as used herein has a lessened responsiveness to IFN (*e.g.*, IFNα, IFNβ and/or IFNγ) as compared with a suitable control cell. This cell has a similar phenotype to a cell in which IFN pathways have been inhibited with an IFN-inhibitory substance, as described above, except that it is a constitutive or inducible property of the cell. In particular embodiments, the cell has a reduced responsiveness to IFN α/β (*e.g.*, via the IFNAR receptor). Alternatively, or additionally, the cell has a reduced responsiveness to IFN- γ (*e.g.*, via the IFN- γ receptor). The reduced responsiveness may be a property of the cell (*e.g.*, Vero cells have a defect in interferon signaling pathways) that may exist in the native state. Alternatively, the cell may be genetically modified to reduce responsiveness to IFN, for example, by modifying the cell by gene disruption of the gene(s) encoding one or more of the IFN receptor proteins or subunits (*e.g.*, the IFNAR protein and/or the α and/or β subunit of the IFNγ receptor), by modifying the cell to stably

express an antisense RNA or inhibitory RNA to at least one of the IFN receptor subunits, or by otherwise modifying the cell so that it has the desired phenotype. The nature of the defect in the IFN response pathway need not be identified or attributable to a single alteration in the cell. For example, mutagenesis or cell selection techniques, as known by those skilled in the art may be employed to produce a cell having the desired characteristics. The cellular responsiveness to IFN (*e.g.*, IFNα, IFNβ and/or IFNγ) is preferably reduced by at least *e.g.*, by at least about 50%, 60%, 75%, 85%, 90%, 95%, 99% or more as compared with a suitable control cell.

In embodiments of the invention, the reduced cellular responsiveness is at least partially attributable to the number of IFN receptors *e.g.*, α/β and/or γ receptors or subunits thereof, on the cell surface being substantially reduced, *e.g.*, by at least about 50%, 60%, 75%, 85%, 90%, 95%, 99% or more as compared with a suitable control cell. Alternatively, and preferably, the cell has been modified such that it "lacks detectable interferon receptors" (*e.g.*, IFNAR and IFNγ receptors). In other embodiments, the cell "lacks detectable interferon α/β (IFNAR) receptors" or "lacks detectable interferon γ" receptors. IFN receptors may be detected by any suitable technique known in the art, including Western blots, ligand binding assays, antibody-based assays, or bioassays measuring biological responses associated with activation of the IFN receptor(s).

As used herein, the terms "express", "expresses", "expressed" or "expression", and the like, with respect to a nucleic acid sequence (*e.g.*, RNA or DNA) indicates that the nucleic acid sequence is transcribed and, optionally, translated. Thus, a nucleic acid sequence may encode a non-translated RNA such as an interfering RNA or an antisense RNA sequence.

II. Alphavirus Vectors.

The present invention may be practiced using alphavirus vectors, more preferably a propagation-incompetent alphavirus vector, still more preferably an alphavirus replicon vector (as described below). Alphavirus vectors, including replicon vectors, are described in U.S. Patent No. 5,505,947 to Johnston et al.; U.S. Patent No. 5,792,462 to Johnston et al.; U.S. Patent No.

5,814,482 to Dubensky et al.; U.S. Patent No. 5,843,723 to Dubensky et al.; U.S. Patent No. 5,789,245 to Dubensky et al.; U.S. Patent No. 5,739,026 to Garoff et al.; the disclosures of which are incorporated herein by reference in their entireties. In embodiments of the invention, the alphavirus vector is a Sindbis (*e.g.*, TR339) or VEE (*e.g.*, having a mutation at nt3 of the genomic RNA following the methylated cap) vector, a Sindbis (*e.g.*, TR339) or VEE (*e.g.*, having a mutation at nt3 of the genomic RNA following the methylated cap) replicon vector, a Sindbis (*e.g.*, TR339) chimeric vector comprising a Sindbis (*e.g.*, TR339) genomic RNA or Sindbis (*e.g.*, TR339) glycoproteins, or a VEE chimeric vector comprising a VEE genomic RNA (*e.g.*, having a mutation at nt3 of the genomic RNA following the methylated cap) or VEE glycoproteins.

Alphavirus vectors elicit a strong host response to immunogen. While not wishing to be held to any particular theory of the invention, it appears that alphavirus vectors induce a more balanced and comprehensive immune response (*i.e.*, cellular and humoral immunity) than do conventional vaccination methods. Moreover, it appears that alphavirus vectors induce a strong immune response, in part, because they directly infect and replicate within dendritic cells. The resulting presentation of antigen to the immune system induces a strong immune response. The alphavirus 26S subgenomic promoter also appears to provide high level of expression of a heterologous nucleic acid encoding antigen.

The alphavirus vectors employed in the present invention may be a chimeric alphavirus, as that term is understood in the art and defined herein. For example, the alphavirus structural proteins may be from one alphavirus (*e.g.*, a Sindbis virus such as TR339) and the genomic RNA packaged within the capsid may be from another alphavirus. Alternatively, the alphavirus virus may be assembled from structural proteins derived from more than one alphavirus.

The alphavirus vector preparation may be partially or highly purified, or may be a relatively crude cell lysate or supernate from a cell culture, as known in the art.

According to the present invention, the alphavirus genomic RNA may comprise a sequence encoding a substance that inhibits cellular responsiveness

to IFN and/or a substance that inhibits cellular PKR pathway activity. The alphavirus genomic RNA may further comprise a sequence encoding one or more heterologous nucleic acids of interest, and/or or a sequence encoding one or more of the alphavirus structural proteins.

A. Double Promoter Vectors.

In embodiments of the invention, the inventive methods may be carried out with an alphavirus double promoter vector. A double promoter vector is typically a replication and propagation competent virus that retains the sequences encoding the alphavirus structural proteins sufficient to produce an alphavirus particle. Double promoter vectors are described in United States Patent No. 5,185,440, 5,505,947 and 5,639,650, the disclosures of which are incorporated in their entireties by reference. Preferred alphaviruses for constructing the double promoter vectors are Sindbis (*e.g.*, TR339) and VEE (*e.g.*, having a mutation at nucleotide 3 of the genomic RNA following the methylated cap) viruses. In addition, the double promoter vector may contain one or more attenuating mutations. Attenuating mutations are described in more detail hereinbelow.

In preferred embodiments, the double promoter vector is constructed so as to contain a second subgenomic promoter (*i.e.*, 26S promoter) inserted 3' to the virus RNA encoding the structural proteins. The heterologous RNA may be inserted between the second subgenomic promoter, so as to be operatively associated therewith, and the 3' UTR of the virus genome. Heterologous RNA sequences of less than about 3 kilobases, more preferably those less than about 2 kilobases, and more preferably still those less than about 1 kilobase, can be inserted into the double promoter vector. In a preferred embodiment of the invention, the double promoter vector is derived from a Sindbis (*e.g.*, TR339) genomic RNA, and the second subgenomic promoter is also a Sindbis (*e.g.*, TR339) subgenomic promoter. In an alternate preferred embodiment, the double promoter vector is derived from a VEE genomic RNA (*e.g.*, having a mutation at nt3 of the genomic RNA), and the second subgenomic promoter is also a VEE subgenomic promoter.

B. Replicon Vectors.

Alphavirus replicon vectors, which are infectious, propagation-defective, virus vectors can also be used to carry out the present invention. Replicon vectors are described in more detail in WO 96/37616 to Johnston et al., U.S. Patent No. 5,505,947 to Johnston et al., and U.S. Patent No. 5,792,462 to Johnston et al. Preferred alphaviruses for constructing the replicon vectors according to the present invention are Sindbis (*e.g.*, TR339) and VEE (*e.g.*, having a mutation at nucleotide 3 of the genomic RNA), and chimeras thereof.

In general, in the replicon system, a foreign nucleic acid to be expressed is inserted in place of at least a portion of one or more of the viral structural protein genes in a transcription vector containing the viral sequences necessary for viral replication (*e.g.*, the nsp1-4 genes). RNA transcribed from this vector contains sufficient viral sequences (*e.g.*, the viral nonstructural genes) responsible for RNA replication and transcription. Thus, if the transcribed RNA is introduced into susceptible cells, it will be replicated and translated to give the replication proteins. These proteins will transcribe the recombinant genomic RNA, including the foreign nucleic acid, which will then be translated to produce high levels of the foreign protein. The autonomously replicating RNA (*i.e.*, replicon) can only be packaged into virus particles if the deleted alphavirus structural protein genes are provided on one or more helper molecules, which are provided to the helper cell, or by helper sequences stably expressed by a packaging cell.

Preferably, the helper molecules do not contain the viral nonstructural genes for replication, but these functions are provided *in trans* by the replicon molecule. The transcriptase functions translated from the replicon molecule transcribe the structural protein genes on the helper molecule, resulting in the synthesis of viral structural proteins and packaging of the replicon into virus-like particles. Preferably, the helper molecules do not contain a functional alphavirus packaging signal. As the alphavirus packaging or encapsidation signal is located within the nonstructural genes, the absence of these sequences in the helper molecules precludes their incorporation into virus particles.

Accordingly, the replicon molecule is "propagation defective" or "propagation incompetent," as described hereinabove. Typically, the resulting alphavirus particles are propagation defective inasmuch as the replicon RNA in these particles does not encode all of the alphavirus structural proteins required for encapsidation, at least a portion of at least one of the required structural proteins being deleted therefrom, such that the replicon RNA initiates only an abortive infection; no new viral particles are produced, and there is no spread of the infection to other cells. Alternatively, the replicon RNA may comprise one or more mutations within the structural protein coding sequences, which interfere(s) with the production of a functional structural protein(s).

Typically, the replicon molecule comprises an alphavirus packaging signal.

The replicon molecule is self-replicating. Accordingly, the replicon molecule comprises sufficient coding sequences for the alphavirus nonstructural polyprotein so as to support self-replication. In embodiments of the invention, the replicon encodes the alphavirus nsP1, nsP2, nsP3 and nsP4 proteins.

The replicon molecules of the invention do not encode one or more of the capsid, E1 or E2 alphavirus structural proteins. By "do(es) not encode" one or more structural proteins, it is intended that the replicon molecule does not encode a functional form of the one or more structural proteins and, thus, a complementing sequence must be provided by a helper or packaging cell to produce new virus particles. In embodiments of the invention, the replicon molecule does not encode a functional form of any of the alphavirus structural proteins.

The replicon may not encode the structural protein(s) because the coding sequence is partially or entirely deleted from the replicon molecule. Alternatively, the coding sequence is otherwise mutated so that the replicon does not express the functional protein. In embodiments of the invention, the replicon lacks all or substantially all of the coding sequence of the structural protein(s) that is not encoded by the replicon, *e.g.*, so as to minimize recombination events with the helper sequences.

In particular embodiments, the replicon molecule may encode at least one, but not all, of the alphavirus structural proteins. For example, the

alphavirus capsid protein may be encoded by the replicon molecule. Alternatively, one or both of the alphavirus glycoproteins may be encoded by the replicon molecule. As a further alternative, the replicon may encode the capsid protein and either the E1 or E2 glycoprotein.

In other embodiments, none of the alphavirus structural proteins are encoded by the replicon molecule. For example, all or substantially all of the sequences encoding the alphavirus capsid protein and glycoproteins may be deleted from the replicon molecule.

In representative embodiments, a composition comprising a population of replicon particles of the invention contains no detectable propagation-competent alphavirus particles. Propagation-competent virus may be detected by any method known in the art, *e.g.*, by neurovirulence following intracerebral injection into suckling mice, or by passage twice on alphavirus-permissive cells (*e.g.*, BHK cells) and evaluation for virus induced cytopathic effects.

Replicon vectors that do not encode the alphavirus capsid protein, may nonetheless comprise a capsid translational enhancer region operably associated with a heterologous sequence, or the sequences encoding the non-structural proteins and/or encoding the alphavirus structural proteins (*e.g.*, E1 and/or E2 glycoproteins) so as to enhance expression thereof. *See, e.g.*, PCT Application No. PCT/US01/27644; U.S. Patent No. 6,224,879 to Sjoberg et al., Smerdou et al., (1999) *J. Virology* **73**:1092; Frolov et al., (1996) *J. Virology* **70**:1182; and Heise et al. (2000) *J. Virol.* **74**:9294-9299 (the disclosures of which are incorporated herein in their entireties).

III. Attenuating Mutations.

The methods of the present invention may also be carried out with alphavirus genomic RNA and particles including attenuating mutations. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide sequence containing a mutation, or an amino acid encoded by a nucleotide sequence containing a mutation, which mutation results in a decreased probability of causing disease in its host (*i.e.*, reduction in virulence), in accordance with standard terminology in the art. *See, e.g.,* B. Davis et al.,

MICROBIOLOGY 132 (3d ed. 1980). The phrase "attenuating mutation" excludes mutations or combinations of mutations which would be lethal to the virus.

Appropriate attenuating mutations will be dependent upon the alphavirus used, and will be known to those skilled in the art. Exemplary attenuating mutations include, but are not limited to, those described in United States Patent No. 5,505,947 to Johnston et al., U.S. Patent No. 5,185,440 to Johnston et al., U.S. Patent No. 5,643,576 to Davis et al., U.S. Patent No. 5,792,462 to Johnston et al., and U.S. Patent No. 5,639,650 to Johnston et al., the disclosures of which are incorporated herein in their entirety by reference.

When the alphavirus structural proteins are from VEE, suitable attenuating mutations may be selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating amino acid, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating amino acid, preferably isoleucine or leucine as E1 amino acid 81; codons at E1 amino acid 253 which specify an attenuating amino acid, preferably serine or threonine as E1 amino acid 253; or the deletion of E3 amino acids 56-69, or a combination of the deletion of E3 amino acids 56-59 together with codons at E1 amino acid 253 which specify an attenuating mutation, as provided above.

Another suitable attenuating mutation is an attenuating mutation at nucleotide 3 of the VEE genomic RNA, *i.e.*, the third nucleotide following the 5' methylated cap (*see, e.g.*, U.S. Patent No. 5,643,576 describing a G→C mutation at nt 3). The mutation may be a G → A, U or C, but is preferably a G→ A mutation.

When the alphavirus structural and/or non-structural proteins are from S.A.AR86, exemplary attenuating mutations in the structural and non-structural proteins include, but are not limited to, codons at nsP1 amino acid position 538

which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid 372 which specify an attenuating amino acid, preferably leucine, at E2 amino acid residue 372; codons at E2 amino acid position 376 which specify an attenuating amino acid, preferably alanine as E2 amino acid 376; in combination, codons at E2 amino acid residues 304, 314, 372 and 376 which specify attenuating amino acids, as described above; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid 96; and codons at nsP2 amino acid position 372 which specify an attenuating amino acid, preferably valine as nsP2 amino acid 372; in combination, codons at nsP2 amino acid residues 96 and 372 which encode attenuating amino acids at nsP2 amino acid residues 96 and 372, as described above; codons at nsP2 amino acid residue 529 which specify an attenuating amino acid, preferably leucine, at nsP2 amino acid residue 529; codons at nsP2 amino acid residue 571 which specify an attenuating amino acid, preferably asparagine, at nsP2 amino acid residue 571; codons at nsP2 amino acid residue 682 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 682; codons at nsP2 amino acid residue 804 which specify an attenuating amino acid, preferably arginine, at nsP2 amino acid residue 804; codons at nsp3 amino acid residue 22 which specify an attenuating amino acid, preferably arginine, at nsP3 amino acid residue 22; and in combination, codons at nsP2 amino acid residues 529, 571, 682 and 804 and at nsP3 amino acid residue 22 which specify attenuating amino acids, as described above.

Other illustrative attenuating mutations include those described in PCT Application No. PCT/US01/27644 (the disclosure of which is incorporated herein in its entirety). For example, the attenuating mutation may be an attenuating mutation at amino acid position 537 of the S.A.AR86 nsP3 protein, more preferably a substitution mutation at this position (*see, e.g.*, **Table 1** below), still more preferably a nonsense mutation that results in substitution of a termination codon. Translational termination (*i.e.*, stop) codons are known

in the art, and include the "opal" (UGA), "amber" (UAG) and "ochre" (UAA) termination codons. In embodiments of the invention, the attenuating mutation results in a Cys→opal substitution at S.A.AR85 nsP3 amino acid position 537.

Further exemplary attenuating mutations include an attenuating insertion mutation following amino acid 385 of the S.A.AR86 nsP3 protein. Preferably, the insertion comprises an insertion of at least 2, 4, 6, 8, 10, 12, 14, 16 or 20 amino acids. In embodiments of the invention, the inserted amino acid sequence is rich in serine and threonine residues (*e.g.*, comprises at least 2, 4, 6, or 8 such sites) that serve as a substrate for phosphorylation by serine/threonine kinases.

Preferably, the attenuating mutation comprising insertion of the amino acid sequence Ile-Thr-Ser-Met-Asp-Ser-Trp-Ser-Ser-Gly-Pro-Ser-Ser-Leu-Glu-Ile-Val-Asp (**SEQ ID NO:1**) following amino acid 385 of nsP3 (*i.e.*, the first amino acid is designated as amino acid 386 in nsP3). In other embodiments of the invention, the insertion mutation comprises insertion of a fragment of **SEQ ID NO:1** that results in an attenuated phenotype. Preferably, the fragment comprises at least 4, 6, 8, 10, 12,14 or 16 contiguous amino acids from **SEQ ID NO:1**.

Those skilled in the art will appreciate that other attenuating insertion sequences comprising a fragment of the sequence set forth above, or which incorporate conservative amino acid substitutions into the sequence set forth above, may be routinely identified by those of ordinary skill in the art (as described above). While not wishing to be bound by any theory of the invention, it appears that the insertion sequence of **SEQ ID NO:1** is highly phosphorylated at serine residues, which confers an attenuated phenotype. Thus, other attenuating insertion sequences which serve as substrates for serine (or threonine) phosphorylation may be identified by conventional techniques known to those skilled in the art.

Alternatively, or additionally, there is a Tyr→Ser substitution at amino acid 385 of the S.A.AR86 nsP3 (*i.e.*, just prior to the insertion sequence above). This sequence is conserved in the non-virulent Sindbis-group viruses, but is not present in S.A.AR86.

Other attenuating mutations for S.A.AR86 include attenuating mutations at those positions that diverge between S.A.AR86 and non-neurovirulent Sindbis group viruses, including attenuating mutations at nsP2 amino acid position 256 (preferably Arg -> Ala), 648 (preferably Ile -> Val) or 651 (preferably Lys -> Glu), attenuating mutations at nsP3 amino acid position 344 (preferably Gly -> Glu), 441 (preferably Asp -> Gly) or 445 (preferably Ile -> Met), attenuating mutations at E2 amino acid position 243 (preferably Ser -> Leu), attenuating mutations at 6K amino acid position 30 (preferably Val -> Ile), and attenuating mutations at E1 amino acid positions 112 (preferably Val -> Ala) or 169 (preferably Leu -> Ser).

Also preferred are alphavirus vectors in which there is an attenuating mutation in the capsid protease that reduces, or even ablates, the autoprotease activity of the capsid and results, therefore, in non-viable virus. Capsid mutations that reduce or ablate the autoprotease activity of the alphavirus capsid are known in the art, *see e.g.*, WO 96/37616 to Johnston et al., the disclosure of which is incorporated herein in its entirety. In particular embodiments, the alphavirus vector comprises a VEE capsid protein in which the capsid protease is reduced or ablated, *e.g.*, by introducing an amino acid substitution at VEE capsid position 152, 174, or 226. Alternatively, one or more of the homologous positions in other alphaviruses may be altered to reduce capsid protease activity.

If the alphavirus vector comprises a Sindbis-group virus (*e.g.*, Sindbis, TR339, S.A.AR86, GirdwoodSA, Ockelbo) capsid protein, the attenuating mutation may be a mutation at capsid amino acid position 215 (*e.g.*, a Ser→Ala) that reduces capsid autoprotease activity (*see*, Hahn et al., (1990) *J. Virology* **64**:3069).

In particular preferred embodiments, the "attenuating" mutation reduces (*e.g.*, by at least 25%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more) the neurovirulence of the alphavirus vector (*e.g.*, as determined by intracerebral injection in weanling or adult mice). In embodiments of the invention, the attenuated virus is an attenuated Sindbis (*e.g.*, TR339) or VEE virus vector, or a chimeric vector as described herein, with reduced neurovirulence.

It is not necessary that the attenuating mutations of the invention eliminate all pathology or adverse effects associated with virus administration, as long as there is some improvement or benefit (*e.g.*, increased safety and/or reduced morbidity and/or reduced mortality) as a result of the attenuating mutation.

In other embodiments of the invention, the attenuating mutation does not result in a significant reduction in expression of a heterologous nucleic acid from the attenuating alphavirus genomic RNA, *i.e.*, expression of a heterologous nucleic acid is essentially the same as in non-attenuated viruses. Alternatively, expression of a heterologous nucleic acid may even be enhanced in the attenuated virus as compared with the non-attenuated virus.

In particular preferred embodiments, the attenuating mutation is an attenuating mutation in one or more of the cleavage domains between the alphavirus nonstructural (nsp) genes, *e.g.*, the nsP1/nsP2 cleavage region, the nsP2/nsP3 cleavage region, and/or the nsP3/nsP4 cleavage region as described in PCT Application No. PCT/US01/27644 (the disclosure of which is incorporated herein in its entirety). An exemplary attenuating mutation is a mutation at S.A.AR86 nsP1 amino acid 538 (position P3), more preferably a substitution mutation at S.A.AR86 nsP1 amino acid 538, still more preferably a Thr→Ile substitution at S.A.AR86 nsP1 amino acid 538.

Likewise, those skilled in the art may identify attenuating mutations other than those specifically disclosed herein using other methods known in the art, *e.g.*, looking at neurovirulence in weanling or adult mice following intracerebral injection. Methods of identifying attenuating mutations in alphaviruses are described by Olmsted et al., (1984) *Science* **225**:424 and Johnston and Smith, (1988) *Virology* **162**:437; the disclosures of which are incorporated herein in their entireties.

To identify other attenuating mutations other than those specifically disclosed herein, amino acid substitutions may be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like.

Amino acid substitutions other than those disclosed herein may be

achieved by changing the codons of the genomic RNA sequence (or a DNA sequence), according to the following codon table:

TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	ACU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In identifying other attenuating mutations, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (*see*, Kyte and Doolittle, (1982) *J. Mol. Biol.* **157**:105;

incorporated herein by reference in its entirety). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, *Id.*), these are:

isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

Accordingly, the hydropathic index of the amino acid (or amino acid sequence) may be considered when identifying additional attenuating mutations according to the present invention.

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Patent No. 4,554,101 (incorporated herein by reference in its entirety) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (±3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); threonine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± l); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

Thus, the hydrophilicity of the amino acid (or amino acid sequence) may be considered when identifying additional attenuating mutations according to the present invention.

Mutations may be introduced into the alphavirus vector by any method known in the art. For example, mutations may be introduced into the alphavirus

RNA by performing site-directed mutagenesis on the cDNA which encodes the RNA, in accordance with known procedures (*see*, Kunkel, *Proc. Natl. Acad. Sci. USA* **82**, 488 (1985), the disclosure of which is incorporated herein by reference in its entirety). Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures.

IV. Helper Cells, Helper Constructs and Methods of Producing Viral Particles.

As another aspect, the present invention also encompasses methods and helper cells for producing alphavirus particles *in vitro*. Methods and helper cells for producing alphavirus stocks, including double-promoter alphaviruses and alphavirus replicon particles are known in the art. *See, e.g.*, Patent No. 5,185,440 to Davis et al., U.S. Patent No. 5,505,947 to Johnston et al.; U.S. Patent No. 5,792,462 to Johnston et al., and Pushko et al. (1997) *Virol.* **239**:389-401; the disclosures of which are incorporated herein by reference in their entireties. Preferred alphaviruses include Sindbis (*e.g.*, TR339), VEE (*e.g.*, having a mutation at nucleotide 3 of the viral genomic RNA following the methylated cap), S.A.AR86, Semliki Forest Virus, and chimeric alphaviruses thereof.

In preferred embodiments, the inventive methods and helper cells are used to produce propagation-incompetent alphavirus particles, more preferably, propagation-incompetent alphavirus replicon particles. According to this embodiment, the helper cells of the invention contain one or more helper nucleic acid sequences (*e.g.*, as DNA and/or RNA molecules) encoding the alphavirus structural proteins (*e.g.*, Sindbis structural proteins). The combined expression of the replicon molecule and the one or more helper molecules in the helper cell results in the production of an assembled alphavirus particle comprising a replicon RNA packaged within a virion comprising alphavirus structural proteins, which is able to infect a cell, but is unable to produce a productive infection (*i.e.*, produce new virus particles). In particular embodiments, the helper sequences are stably incorporated into (*e.g.*, into the

chromosome or carried by a stable episome) the helper cell, *i.e.*, to produce a stable packaging cell.

In embodiments of the invention, the population of alphavirus particles produced according to the invention contains no detectable propagation-competent alphavirus particles. Propagation-competent virus may be detected by any method known in the art, *e.g.*, by neurovirulence following intracerebral injection into suckling mice, or by passage twice on alphavirus-permissive cells (*e.g.*, BHK cells) and evaluation for virus induced cytopathic effects.

Thus, according to one embodiment of the invention, the present invention provides a method of producing a recombinant alphavirus particle *in vitro*, comprising: providing to a cell: (a) a recombinant alphavirus genomic RNA, comprising (i) sequences encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) a competent alphavirus packaging sequence; (b) sequences encoding the alphavirus structural proteins sufficient for producing an alphavirus particle; (c) a substance that inhibits PKR pathway activity in the cell; and/or (d) a substance that inhibits cellular responsiveness to IFN; wherein the combined expression of the alphavirus replicon RNA and the sequences encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and producing an alphavirus particle in the cell.

The substance that inhibits PKR pathway activity in the cell is as defined hereinabove. The substance may be provided directly to the cell and/or may be expressed from a nucleic acid sequence in the cell, *e.g.*, from a DNA or RNA sequence, from a helper sequence encoding one or more of the alphavirus structural proteins, from an alphavirus genomic RNA, from a DNA molecule integrated into the genomic DNA, from an episome, or from any vector known in the art. For example, the substance may be expressed from the recombinant alphavirus genomic RNA expressing a heterologous nucleic acid of interest.

Likewise, the substance that inhibits cellular responsiveness to IFN is as defined hereinabove. The substance may also be provided directly to the

cell and/or may be expressed from a nucleic acid sequence in the cell, *e.g.*, from a DNA or RNA sequence, from a helper sequence encoding one or more of the alphavirus structural proteins, from an alphavirus genomic RNA, from a DNA molecule integrated into the genomic DNA, from an episome, or from any vector known in the art. For example, the substance may be expressed from the recombinant alphavirus genomic RNA expressing a heterologous nucleic acid of interest.

The substance that inhibits cellular responsiveness to IFN and the substance that inhibits PKR pathway activity in the cell may be provided together, *e.g.*, within the same composition or expressed from a single nucleic acid molecule.

In other embodiments of the invention, the method employs a cell that has the desired property of reduced responsiveness to IFN, a reduced PKR pathway activity, or both. According to this embodiment, the present invention provides a method of producing a recombinant alphavirus particle, comprising: providing to a cell permissive for alphavirus replication, said cell having a reduced responsiveness to IFN, a reduced PKR pathway activity, or both (a) a recombinant alphavirus genomic RNA comprising (i) sequences encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) a competent alphavirus packaging sequence; and (b) sequences encoding the alphavirus proteins sufficient to produce an alphavirus particle; wherein the combined expression of the alphavirus replicon RNA and the sequences encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and producing an alphavirus particle in the cell.

Cells having reduced responsiveness to IFN and/or reduced PKR pathway activity are as described hereinabove. In particular preferred embodiments, the cell is a Vero cell. In other embodiments, gene disruption has been used to knock out the gene(s) encoding one or more of the IFN receptor proteins or subunits (*e.g.*, the IFNAR receptor and/or the IFNγ receptor). Alternatively, expression of one or more of the receptor proteins or subunits may be inhibited with an antisense RNA or an interfering RNA

molecule transiently or stably expressed (*e.g.*, from the cellular DNA or an episome). In particular embodiments, the cell does not produce detectable IFNAR receptors and/or detectable IFNγ receptors, as described above. Alternatively, no detectable IFN receptors may be present on the cell.

Likewise, in embodiments of the invention, gene disruption is used to knock out the gene(s) encoding PKR in the cell. Alternatively, expression of the PKR protein may be inhibited with an antisense RNA or an interfering RNA molecule transiently or stably expressed (*e.g.*, from the cellular DNA or an episome). In particular embodiments, the cell produces no detectable PKR activity, either constitutively and/or in response to IFN stimulation of the cell.

The methods of the invention may be carried out to achieve a higher yield of alphavirus particles. The number of alphavirus particles produced per cell may be increased by as much as about 2, 5, 10, 50, 100, 1000, 2500, 5000 or 10,000 fold or higher as compared with a suitable control cell (*e.g.*, in the absence of the gene disruption, the inhibitory substance(s) and the like). Likewise, the present invention may achieve similar elevations in transgene expression from a recombinant alphavirus vector.

Moreover, the kinetics of alphavirus production may be altered so that higher levels of alphavirus production are observed at earlier time points (*e.g.*, at about 2, 4, 6, 8, 10, 12, 18, 20 or 24 hours post-infection), in particular in those aspects of the invention in which the cellular PKR pathway activity is inhibited or reduced.

Where the invention is carried out to make a stock of alphavirus replicon particles, the helper cell comprises helper sequences which may be provided as an RNA or DNA molecule(s), or may be stably expressed by the cell by integration into the cellular DNA or from an episome. In other embodiments of the invention, the helper cell further comprises one or more replicon molecules each comprising one or more heterologous nucleotide sequences, as described herein.

Typically, the helper sequences will not include an alphavirus packaging sequence, whereas the replicon will contain an alphavirus packaging sequence (*e.g.*, a Sindbis packaging sequence).

The helper cells are typically alphavirus-permissive cells. Alphavirus-permissive cells employed in the methods of the present invention are cells that, upon transfection with the viral RNA transcript, are capable of producing viral particles. According to the present invention, the cells may be rendered alphavirus permissive by interfering with the normal IFN and/or PKR induced antiviral state of the cell. Preferred alphavirus-permissive cells are Sindbis permissive cells (*e.g.*, TR339-permissive cells), VEE permissive cells, S.A.AR86-permissive cells, and Semliki Forest virus-permissive cells. Alphaviruses have a broad host range. Examples of suitable host cells include, but are not limited to fibroblasts, Vero cells, baby hamster kidney (BHK) cells, 293 cells, 293T cells, and chicken embryo fibroblast cells (*e.g.*, DF-1 cells).

As a further aspect, the present invention provides helper cells with reduced susceptibility to IFN and/or PKR-induced antiviral state. According to this embodiment, the helper cell comprises a sequence that encodes a substance that inhibits cellular responsiveness to IFN and/or reduces PKR pathway activity in the cell, each as described above.

Typically, for the production of alphavirus replicon particles, the helper cell will further comprise a sequence encoding at least one of the alphavirus structural proteins as described below. Thus, in this embodiment, the present invention further provides a helper cell for producing an alphavirus particle, comprising: (a) a sequence encoding an alphavirus structural protein(s); and (b) a sequence encoding a substance that inhibits cellular PKR pathway activity and/or (c) a sequence encoding a substance that inhibits cellular responsiveness to IFN. In particular embodiments, the sequences of (b) and (c) are stably expressed by the cell by integration into the cellular DNA or from an episome. The sequences encoding the alphavirus structural protein(s) may be present in the cell as described above *e.g.*, as an RNA molecule(s), DNA molecule(s), as part of the replicon, and the like. The sequences encoding the alphavirus structural protein(s) may further encode the sequences of (b) and/or (c), also as described above.

As an alternative embodiment, the present invention provides a helper cell for producing an alphavirus particle, wherein the cell has a reduced responsiveness to IFN and/or a reduced PKR pathway activity (each as

described above) comprising (a) a sequence encoding an alphavirus structural protein(s); and (b) a sequence encoding a substance that inhibits cellular PKR pathway activity and/or a sequence encoding a substance that inhibits cellular responsiveness to IFN. In particular embodiments, the cell has substantially reduced levels of detectable IFNAR receptors, IFNγ receptors, or both. In other embodiments, the cells may lack any detectable IFNAR receptors, IFNγ receptors, or both. Alternatively, or additionally, the helper cell may further contain substantially reduced levels of PKR activity, or may lack any detectable PKR activity.

In particular embodiments, the helper cells of the invention may further comprise sequences encoding the alphavirus structural proteins sufficient to produce an alphavirus particle, as described herein. Alternatively, or additionally, the helper cell may comprise a replicon RNA (*e.g.*, as an RNA molecule or expressed from DNA) comprising one or more heterologous sequences, also as described herein.

As described hereinabove, in the production of a replicon particle, sequences encoding the alphavirus structural proteins are distributed among one or more helper molecules (*e.g.*, two or three helper RNAs). In addition, one or more structural proteins may be encoded by the replicon RNA, provided that the replicon RNA does not encode at least one structural protein such that the resulting alphavirus particle is propagation-incompetent in the absence of the helper sequence(s).

In illustrative embodiments, at least one of the alphavirus structural and/or non-structural proteins encoded by the replicon and helper molecules contain one or more attenuating mutations, as described herein.

The helper sequences and/or replicon may further comprise an alphavirus capsid enhancer sequence (*e.g.*, a S.A.AR86 capsid enhancer sequence), as described above. *See, e.g.*, PCT Application No. PCT/US01/27644; U.S. Patent No. 6,224,879 to Sjoberg et al., Smerdou et al., (1999) *J. Virology* **73**:1092; Frolov et al., (1996) *J. Virology* **70**:1182; and Heise et al. (2000) *J. Virol.* **74**:9294-9299 (the disclosures of which are incorporated herein in their entireties).

In one particular embodiment, the replicon molecule encodes at least one, but not all, of the alphavirus structural proteins (*e.g.*, the E1 and/or E2 glycoproteins and/or the capsid protein). According to representative aspects of this embodiment, the replicon encodes the capsid protein, and the E1 and E2 glycoproteins are encoded by one or more separate helper molecules. In other particular embodiments, the glycoproteins are encoded by two separate helper molecules, so as to minimize the possibility of recombination to produce replication-competent virus.

In another illustrative embodiment, the replicon does not encode any of the structural proteins (*e.g.*, the E1 glycoprotein, the E2 glycoprotein, and the capsid protein). According to this embodiment, the capsid protein and alphavirus glycoproteins are encoded by one or more helper molecules, preferably two or more helper molecules. By distributing the coding sequences for the structural proteins among two, three or even more helper molecules, the likelihood that recombination will result in replication-competent virus is reduced.

In a further embodiment, the replicon does not encode any of the alphavirus structural proteins, and may lack the sequences encoding the alphavirus structural proteins.

As described above, the replicon may not encode the structural protein(s) because of a partial or complete deletion of the coding sequence(s) or otherwise contains a mutation that prevents the expression of a functional protein(s). In embodiments of the invention, all or substantially all of the coding sequences for the structural protein(s) that is not encoded by the replicon are deleted from the replicon molecule.

In one embodiment, the E1 and E2 glycoproteins are encoded by one helper molecule, and the capsid protein is encoded by another helper molecule. In another embodiment, the E1 glycoprotein, E2 glycoprotein, and capsid protein are each encoded by separate helper molecules. In other embodiments, the capsid protein and one of the glycoproteins are encoded by one helper molecule, and the other glycoprotein is encoded by a second helper molecule.

In some embodiments of the invention, duplicate copies of the structural proteins are not present among the replicon and helper molecules. If duplicate

copies of the structural proteins are present, other approaches can be used to avoid recombination events and/or generation of replication-competent virus.

In other particular embodiments, the helper and replicon sequences are RNA molecules that are introduced into the cell, *e.g.*, by lipofection or electroporation. Uptake of helper RNA and replicon RNA molecules into packaging cells *in vitro* can be carried out according to any suitable means known to those skilled in the art. Uptake of RNA into the cells can be achieved, for example, by treating the cells with DEAE-dextran, treating the RNA with LIPOFECTIN™ before addition to the cells, or by electroporation, with electroporation being the currently preferred means. These techniques are well known in the art. *See e.g.*, United States Patent No. 5,185,440 to Davis et al., and PCT Publication No. WO 92/10578 to Bioption AB, the disclosures of which are incorporated herein by reference in their entirety.

Alternatively, one or all of the helper and/or replicon molecules are DNA molecules, which are preferably stably integrated into the genome of the helper cell or expressed from an episome (*e.g.*, an EBV derived episome). The DNA molecule may be any vector known in the art, including but not limited to a non-integrating DNA vector, such as a plasmid, or a viral vector.

The present invention further provides helper constructs, as described above, comprising sequences that encode one or more of the alphavirus structural proteins and a substance that inhibits cellular responsiveness to IFN and/or a substance that inhibits PKR pathway activity in the cell. The helper sequence may be an RNA or DNA sequence and may further be carried by a vector, as described above, or may be integrated into the cellular DNA.

V. Recombinant Alphavirus Vectors.

According to embodiments of the invention, it is desirable to employ an alphavirus vector that encodes one or more (*e.g.*, two, three, four, five, *etc.*) heterologous nucleic acid sequences, preferably encoding a polypeptide of interest. In particular embodiments, wherein there are two or more heterologous nucleotide sequences, each heterologous nucleic acid sequence will typically be operably associated with a promoter. Alternatively, an internal ribosome entry site (IRES) sequence(s) can be placed downstream of a promoter and upstream

of the heterologous nucleic acid sequence(s). As a further alternative, the polypeptide encoded by the heterologous sequence may be expressed essentially as a polyprotein (*i.e.*, fused to another viral protein, *e.g.*, a structural or nonstructural protein), separated by an intervening cleavage site, such as the foot and mouth disease virus (FMDV) 2a protease. The FMDV protease will auto-catalytically cleave the polyprotein to generate the polypeptide encoded by the heterologous nucleic acid sequence. The heterologous nucleic acid sequences may be associated with a constitutive or inducible promoter, *e.g.*, such as an alphavirus 26S subgenomic promoter. In particular embodiments, the Sindbis 26S subgenomic promoter is suitable for use with Sindbis replication proteins, and the VEE 26S subgenomic promoter can be employed with VEE replication proteins, and the like.

Heterologous nucleic acids of interest include nucleic acids encoding peptides and proteins, preferably immunogenic (*e.g.*, for an immunogenic composition or a vaccine) or therapeutic (*e.g.*, for medical or veterinary uses) polypeptides.

An "immunogenic" polypeptide, or "immunogen" as used herein is any polypeptide that elicits an immune response in a subject, more preferably, the immunogenic polypeptide is suitable for providing some degree of protection to a subject against a disease. The present invention may be employed to express an immunogenic polypeptide in a subject (*e.g.*, for vaccination) or for immunotherapy (*e.g.*, to treat a subject with cancer or tumors).

An immunogenic polypeptide, or immunogen, may be any polypeptide suitable for protecting the subject against a disease, including but not limited to microbial, bacterial, protozoal, parasitic, and viral diseases. For example, the immunogen may be an orthomyxovirus immunogen (*e.g.*, an influenza virus immunogen, such as the influenza virus hemagglutinin (HA) surface protein or the influenza virus nucleoprotein gene, or an equine influenza virus immunogen), or a lentivirus immunogen (*e.g.*, an equine infectious anemia virus immunogen, a Simian Immunodeficiency Virus (SIV) immunogen, or a Human Immunodeficiency Virus (HIV) immunogen, such as the HIV or SIV envelope GP160 protein, the HIV or SIV matrix/capsid proteins, and the HIV or SIV gag, pol and env genes products). The immunogen may also be an

arenavirus immunogen (*e.g.*, Lassa fever virus immunogen, such as the Lassa fever virus nucleocapsid protein gene and the Lassa fever envelope glycoprotein gene), a Picornavirus immunogen (*e.g.*, a Foot and Mouth Disease virus immunogen), a poxvirus immunogen (*e.g.*, vaccinia, such as the vaccinia L1 or L8 genes), an Orbivirus immunogen (*e.g.*, an African horse sickness virus immunogen), a flavivirus immunogen (*e.g.*, a yellow fever virus immunogen, a West Nile virus immunogen, or a Japanese encephalitis virus immunogen), a filovirus immunogen (*e.g.*, an Ebola virus immunogen, or a Marburg virus immunogen, such as NP and GP genes), a bunyavirus immunogen (*e.g.*, RVFV, CCHF, and SFS immunogens), or a coronavirus immunogen (*e.g.*, an infectious human coronavirus immunogen, such as the human coronavirus envelope glycoprotein gene, or a porcine transmissible gastroenteritis virus immunogen, or an avian infectious bronchitis virus immunogen). The immunogen may further be a polio antigen, herpes antigen (*e.g.*, CMV, EBV, HSV antigens) mumps antigen, measles antigen, rubella antigen, diptheria toxin or other diptheria antigen, pertussis antigen, hepatitis (*e.g.*, hepatitis A or hepatitis B) antigen, or any other vaccine antigen known in the art.

The present invention may also be advantageously employed to produce an immune response against chronic or latent infective agents, which typically persist because they fail to elicit a strong immune response in the subject. Illustrative latent or chronic infective agents include, but are not limited to, hepatitis B, hepatitis C, Epstein-Barr Virus, herpes viruses, human immunodeficiency virus, and human papilloma viruses. Alphavirus vectors encoding antigens from these infectious agents may be administered to a cell or a subject according to the methods described herein.

Alternatively, the immunogen may be any tumor or cancer cell antigen. Preferably, the tumor or cancer antigen is expressed on the surface of the cancer cell. Exemplary cancer antigens for specific breast cancers are the HER2 and BRCA1 antigens. Other illustrative cancer and tumor cell antigens are described in S.A. Rosenberg, (1999) *Immunity* **10**:281) and include, but are not limited to: MART-1/MelanA, gp100, tyrosinase, TRP-1, TRP-2, MAGE-

1, MAGE-3, GAGE-1/2, BAGE, RAGE, NY-ESO-1, CDK-4, β-catenin, MUM-1, Caspase-8, KIAA0205, HPVE&, SART-1, PRAME, p15, and p53 antigens.

The immunogen may also be a "universal" or "artificial" cancer or tumor cell antigen as described in international patent publication WO 99/51263, which is hereby incorporated by reference in its entirety.

Preferably, administration of an alphavirus virus comprising one or more heterologous nucleotide sequences encoding an immunogen elicits an active immune response in the subject, more preferably a protective immune response (each as defined below).

The term "cancer" has its understood meaning in the art, for example, an uncontrolled growth of tissue that has the potential to spread to distant sites of the body (*i.e.*, metastasize). Exemplary cancers include, but are not limited to, leukemias, lymphomas, colon cancer, renal cancer, liver cancer, breast cancer, lung cancer, prostate cancer, ovarian cancer, melanoma, and the like. Other illustrative cancers include cancers of the bone and bone marrow. Preferred are methods of treating and preventing tumor-forming cancers. The term "tumor" is also understood in the art, for example, as an abnormal mass of undifferentiated cells within a multicellular organism. Tumors can be malignant or benign. Preferably, the inventive methods disclosed herein are used to prevent and treat malignant tumors.

Cancer and tumor antigens according to the present invention have been described hereinabove. Alphaviruses encoding cancer or tumor antigens may be administered in methods of treating cancer or tumors, respectively.

By the terms "treating cancer" or "treatment of cancer", it is intended that the severity of the cancer is reduced or the cancer is at least partially eliminated. Preferably, these terms indicate that metastasis of the cancer is reduced or at least partially eliminated. It is further preferred that these terms indicate that growth of metastatic nodules (*e.g.*, after surgical removal of a primary tumor) is reduced or at least partially eliminated. By the terms "prevention of cancer" or "preventing cancer" it is intended that the inventive methods at least partially eliminate or reduce the incidence or onset of cancer.

Alternatively stated, the present methods slow, control, decrease the likelihood or probability, or delay the onset of cancer in the subject.

Likewise, by the terms "treating tumors" or "treatment of tumors", it is intended that the severity of the tumor is reduced or the tumor is at least partially eliminated. Preferably, these terms are intended to mean that metastasis of the tumor is reduced or at least partially eliminated. It is also preferred that these terms indicate that growth of metastatic nodules (*e.g.*, after surgical removal of a primary tumor) is reduced or at least partially eliminated. By the terms "prevention of tumors" or "preventing tumors" it is intended that the inventive methods at least partially eliminate or reduce the incidence or onset of tumors. Alternatively stated, the present methods slow, control, decrease the likelihood or probability, or delay the onset of tumors in the subject.

It is known in the art that immune responses may be enhanced by immunomodulatory cytokines (*e.g.*, α-interferon, β-interferon, γ-interferon, ω-interferon, τ-interferon, interleukin-1α, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell Growth factor, CD40 Ligand, tumor necrosis factor-α, tumor necrosis factor-β, monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, and lymphotoxin). Accordingly, in particular embodiments of the invention, immunomodulatory cytokines (preferably, CTL inductive cytokines) are administered to a subject in conjunction with the methods described herein for producing an immune response or providing immunotherapy.

Cytokines may be administered by any method known in the art. Exogenous cytokines may be administered to the subject, or alternatively, a nucleotide sequence encoding a cytokine may be delivered to the subject using a suitable vector, and the cytokine produced *in vivo*. In preferred embodiments, an alphavirus vector encoding a cytokine is used to deliver the cytokine to the subject.

The present invention further finds use in methods of producing antibodies *in vivo* for passive immunization techniques. According to this

embodiment an alphavirus vector expressing an immunogen of interest is administered to a subject, as described herein by direct administration or *ex vivo* cell manipulation techniques. The antibody may then be collected from the subject using routine methods known in the art. The invention further finds use in methods of producing antibodies against an immunogen expressed from an alphavirus vector for any other purpose, *e.g.*, for diagnostic purpose or for use in histological techniques.

Alternatively, the present invention may be practiced to express a therapeutic polypeptide in the cell, *in vitro* or *in* vivo. A "therapeutic" polypeptide is a polypeptide that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic" polypeptide is one that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects.

Therapeutic peptides and proteins include, but are not limited to, CFTR (cystic fibrosis transmembrane regulator protein), dystrophin (including the protein product of dystrophin mini-genes, *see, e.g*, Vincent *et al.*, (1993) *Nature Genetics* **5**:130), utrophin (Tinsley *et al.*, (1996) *Nature* **384**:349), clotting factors (Factor XIII, Factor IX, Factor X, *etc.)*, erythropoietin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, hormones, growth factors (*e.g.*, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor –3 and –4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor-α and –β, and the like), cytokines (*e.g.*, α-interferon, β-interferon, γ-interferon, ω-interferon, τ-interferon, interleukin-1α, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell Growth factor, CD40 Ligand, tumor necrosis factor-α, tumor necrosis factor-β, monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, lymphotoxin), suicide

gene products (*e.g.*, herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (*e.g.*, p53, Rb, Wt-1, NF1, VHL, APC, and the like), and any other polypeptide that has a therapeutic effect in a subject in need thereof.

Further exemplary therapeutic polypeptides include those that may used in the treatment of a disease condition including, but not limited to, cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease, Parkinson's disease, Huntington's disease, amylotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (*e.g.*, Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), and diseases of solid organs (*e.g.*, brain, liver, kidney, heart).

The present invention may be preferably, and advantageously, employed for relatively short-term therapeutic regimes. For example, immunomodulators (*e.g.*, cytokines, as defined above) may be delivered using an alphavirus vector as described herein. Likewise, supportive therapeutic agents (*e.g.*, immunomodulators, erythropoietin) may be provided in conjunction with chemotherapy.

As a further alternative, the alphavirus vector may be used to express an antibody against a defective or over-expressed protein. Subjects expressing the defective protein or over-expressed protein may be administered an alphavirus vector according to the invention expressing an antibody that modulates the activity of the protein.

As a further alternative, the heterologous nucleic acid sequence may encode a reporter polypeptide (*e.g.*, an enzyme). Reporter proteins are known in the art and include, but are not limited to, Green Fluorescent Protein, β-galactosidase, alkaline phosphatase, chloramphenicol acetyltransferase, and the like.

Alternatively, in particular embodiments of the invention, the nucleic acid of interest may encode an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (Puttaraju *et al.*, (1999) *Nature Biotech.* **17**:246), or other non-translated RNAs, such as "guide" RNAs (Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* **95**:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*), interfering RNAs, and other nontranslated RNAs.

In general, "antisense" refers to the use of small, synthetic oligonucleotides to inhibit gene expression by inhibiting the function of the target mRNA containing the complementary sequence. Milligan, J.F. et al., *J. Med. Chem.* 36(14), 1923-1937 (1993). Gene expression is inhibited through hybridization to coding (sense) sequences in a specific mRNA target by hydrogen bonding according to Watson-Crick base pairing rules. The mechanism of antisense inhibition is that the exogenously applied oligonucleotides decrease the mRNA and protein levels of the target gene. Milligan, J.F. et al., *J. Med. Chem.* 36(14), 1923-1937 (1993). *See also* Helene, C. and Toulme, J., *Biochim. Biophys. Acta* 1049, 99-125 (1990); Cohen, J.S., Ed., OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION, CRC Press:Boca Raton, FL (1987).

The antisense sequence may also be expressed from a sense sequence within the cell.

Antisense oligonucleotides may be of any suitable length, depending on the particular target being bound. The only limits on the length of the antisense oligonucleotide is the capacity of the virus for inserted heterologous RNA. Antisense oligonucleotides may be complementary to the entire mRNA transcript of the target gene or only a portion thereof. Preferably the antisense oligonucleotide is directed to an mRNA region containing a junction between intron and exon. Where the antisense oligonucleotide is directed to an intron/exon junction, it may either entirely overlie the junction or may be sufficiently close to the junction to inhibit splicing out of the intervening exon during processing of precursor mRNA to mature mRNA (*e.g.*, with the 3' or 5' terminus of the antisense oligonucleotide being positioned within about, for

example, 10, 5, 3 or 2 nucleotides of the intron/exon junction). Also preferred are antisense oligonucleotides which overlap the initiation codon.

The heterologous nucleic acid may be operably associated with expression control elements, such as transcription/translation control signals, an origin of replication, polyadenylation signals, an internal ribosome entry site (IRES), promoters, enhancers, and the like. Those skilled in the art will appreciate that a variety of promoter/enhancer elements may be used depending on the level and tissue-specific expression desired. The promoter/enhancer may be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer may be native or foreign and can be a natural or a synthetic sequence.

Promoters/enhancers that are native to the subject to be treated are most preferred. Also preferred are promoters/enhancers that are native to the heterologous nucleic acid sequence. The promoter/enhancer is chosen so that it will function in the target cell(s) of interest. Mammalian promoters/enhancers are also preferred.

Preferably, the heterologous nucleotide sequence is operably associated with a promoter that provides high level expression of the heterologous nucleotide sequence, *e.g.*, an alphavirus subgenomic 26S promoter (preferably, a VEE, Sindbis or TR339 26S subgenomic promoter).

Expression control elements that are functional in a cell type of interest can also be used according to the present invention. Representative promoters are operable in cells and tissues including, but not limited to, muscle (including cardiac, skeletal and/or smooth muscle), neural tissues (including brain), eye (including retina and cornea), liver, bone marrow, pancreas, spleen, lung, epithelial cells, endothelial cells, macrophages, dendritic cells and other antigen presenting cells.

Inducible expression control elements are preferred in those applications in which it is desirable to provide regulation over expression of the heterologous nucleic acid sequence(s). Inducible promoters/enhancer elements for gene delivery can also be a tissue-specific promoter/enhancer elements, and include muscle specific (including cardiac, skeletal and/or smooth muscle), neural tissue specific (including brain-specific), eye

(including retina-specific and cornea-specific), liver specific, bone marrow specific, pancreatic specific, spleen specific, and lung specific promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metalothionein promoter.

In embodiments of the invention in which the heterologous nucleic acid sequence(s) will be transcribed and then translated in the target cells, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

V. DNA Sequences, Vectors and Transformed Cells.

As a further aspect, the present invention provides DNA sequences (*e.g.*, cDNA sequences) and vectors encoding infectious alphavirus genomic RNA transcripts (*e.g.*, Sindbis genomic transcripts) according to the present invention. In particular embodiments, the DNA encodes and the alphavirus genomic RNA sequence comprise one or more heterologous nucleotide sequences. Also provided are alphavirus particles containing the genomic RNA transcribed from the DNA molecules.

The alphavirus genomic transcript further comprises a sequence encoding a substance that inhibits cellular responsiveness to IFN and/or a sequence encoding a substance that reduces PKR pathway activity in a cell, each substance as described above. According to this embodiment, the alphavirus genomic transcript may comprise a heterologous nucleotide sequence of interest. Alternatively, or additionally, the alphavirus genomic construct comprises a sequence encoding one or more of the alphavirus structural proteins. In one particular embodiment, the alphavirus genomic construct does not encode all of the alphavirus structural proteins (and preferably lacks sequences encoding the alphavirus structural proteins) and comprises (i) a heterologous nucleotide sequences of interest; and (ii) a

sequence encoding a substance that reduces PKR pathway activity in a cell and/or a substance that inhibits cellular responsiveness to interferon.

The present invention further provides vectors and constructs comprising a DNA sequence encoding the alphavirus genomic RNA transcript described above operably associated with a promoter that drives transcription of the DNA sequence. Examples of promoters which are suitable for use with the DNA sequences of the present invention include, but are not limited to T3 promoters, T7 promoters, cytomegalovirus (CMV) promoters, and SP6 promoters.

The DNA sequence may be embedded within any suitable vector known in the art, including but not limited to, plasmids, naked DNA vectors, yeast artificial chromosomes (yacs), bacterial artificial chromosomes (bacs), phage, viral vectors, and the like. Preferably, the vector is a plasmid.

Genomic RNA transcripts may be synthesized from the DNA template by any method known in the art. Preferably, the RNA is synthesized from the DNA sequence *in vitro* using purified RNA polymerase in the presence of ribonucleotide triphosphates and cap analogs in accordance with conventional techniques. Alternatively, the RNA may be synthesized intracellularly after introduction of the DNA.

Further provided are cells containing the DNA sequences, genomic RNA transcripts from the DNA sequences, and alphavirus vectors of the invention. Exemplary cells include, but are not limited to, fibroblast cells, Vero cells, Baby Hamster Kidney (BHK) cells, Chinese Hamster Ovary (CHO) cells, macrophages, dendritic cells, and the like.

The alphavirus DNA constructs, alphavirus genomic RNA transcripts, and virus particles produced therefrom are useful for the preparation of pharmaceutical formulations, such as vaccines. These reagents may further be used in methods of producing virus stocks or polypeptides, either *in vitro* or *in vivo* (typically, *in vitro*). In addition, the DNA clones, genomic RNA transcripts, and infectious viral particles of the present invention are useful for administration to animals for the purpose of producing antibodies to the alphavirus, which antibodies may be collected and used in known diagnostic techniques for the detection of the alphavirus. Antibodies can also be generated to the viral proteins expressed from the DNAs disclosed herein. As another aspect of the

present invention, the claimed DNA clones are useful as nucleotide probes to detect the presence of alphavirus transcripts.

VI. Gene Transfer Technology.

The methods of the present invention provide a means for delivering heterologous nucleic acid sequences into a broad range of host cells. The vectors and other reagents, methods and pharmaceutical formulations of the present invention are additionally useful in a method of administering a polypeptide to a subject in need thereof, as a method of treatment or otherwise. In this manner, the polypeptide may thus be produced *in vivo* in the subject. The subject may be in need of the polypeptide because the subject has a deficiency of the polypeptide, or because the production of the polypeptide in the subject may impart some therapeutic effect, and as explained further below.

In general, the present invention may be employed to deliver any foreign nucleic acid with a biological effect to treat or ameliorate the symptoms associated with any disorder related to gene expression. Illustrative disease states include, but are not limited to: cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (*e.g.*, Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), diseases of solid organs (*e.g.*, brain, liver, kidney, heart), and the like.

Gene transfer has substantial potential use in understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In some cases, the function of these cloned genes is known. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, at least sometimes involving regulatory or structural proteins, which are

inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer may be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the deficiency or imbalance that causes the disease or makes it more severe. The use of site-specific integration of nucleic sequences to cause mutations or to correct defects is also possible.

The instant invention may also be employed to provide an antisense nucleic acid to a cell *in vitro* or *in vivo*. Expression of the antisense nucleic acid in the target cell diminishes expression of a particular protein by the cell. Accordingly, antisense nucleic acids may be administered to decrease expression of a particular protein in a subject in need thereof. Antisense nucleic acids may also be administered to cells *in vitro* to regulate cell physiology, *e.g.*, to optimize cell or tissue culture systems. The present invention is also useful to deliver other non-translated RNAs, *e.g.*, ribozymes (*e.g.*, as described in U.S. Patent No. 5,877,022), interfering RNAs, RNAs that effect spliceosome-mediated *trans*-splicing (Puttaraju *et al.*, (1999) *Nature Biotech.* **17**:246), or "guide" RNAs (*see, e.g.*, Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* **95**:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*) to a target cell.

Delivery of a nucleotide sequence to cells *in vitro* or *in vivo* may further be used for the purpose of producing a polypeptide encoded by the nucleotide sequence, *e.g.*, in an *in vitro* production system or by using an animal expressing the polypeptide as a bioreactor and harvesting the polypeptide therefrom (*e.g.*, in the plasma or milk).

Further, the instant invention finds further use in diagnostic and screening methods, whereby a gene of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

VII. Methods of Nucleic Acid Delivery for *in vitro* and *in vivo* Use.

The present invention may be practiced to deliver a nucleic acid of interest to a cell *in vitro* or *in vivo*. Delivery of a recombinant alphavirus genomic RNA comprising a nucleic acid sequence of interest to a cell *in vitro* and/or *in vivo*, wherein cellular responsiveness to IFN and/or cellular PKR pathway activity is reduced or inhibited, may advantageously result in higher levels of expression of the nucleic acid of interest by the cell. Moreover, the host range of the alphavirus vector may be expanded when cellular responsiveness to IFN and/or cellular PKR pathway activity is reduced or inhibited. For example, the present invention may be used to deliver a recombinant Sindbis vector to a dendritic cell or macrophage for the purpose of expressing the heterologous sequence in the cell, whereas Sindbis replication is substantially limited or even absent in these cells in the presence of intact IFN and/or PKR pathway activity.

Accordingly, as one aspect, the present invention provides a method of delivering a nucleic acid sequence to a cell, comprising: introducing into a cell (a) a recombinant alphavirus genomic RNA comprising (i) sequences encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) a competent alphavirus packaging sequence; (b) a substance that inhibits cellular PKR pathway activity; and/or (c) a substance that inhibits cellular responsiveness to IFN; wherein the heterologous RNA sequence is introduced into and expressed in the cell.

As a further aspect, the present invention provides a method of delivering a nucleic acid sequence to a cell, comprising: introducing into a cell having a reduced responsiveness to IFN or a reduced PKR pathway activity, or both: a recombinant alphavirus genomic RNA comprising (i) sequences encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) a competent alphavirus packaging sequence; wherein the heterologous RNA sequence is expressed in the cell. If the cell has a reduced responsiveness to IFN, the cell may further be provided with a substance that inhibits PKR pathway activity in the cell. Conversely, if the cell has a reduced PKR pathway activity, the cell may further be provided with a substance that inhibits cellular responsiveness to IFN.

A nucleic acid of interest may be delivered to a cell for the purpose of producing the polypeptide encoded thereby, the polypeptide then being collected and, optionally, purified. In an *in vitro* polypeptide production system, an alphavirus genomic RNA encoding the polypeptide is delivered to the cell. The genomic RNA may be provided in the form of a recombinant propagation-competent alphavirus particle. Alternatively, the recombinant alphavirus genomic RNA may be provided as a propagation-incompetent particle (*e.g.*, a replicon particle) or introduced as an RNA molecule (or DNA molecule encoding the same), *e.g.*, by lipofection, electroporation or other standard cell transformation techniques.

Any suitable cell in culture may be used to carry out this aspect of the present invention, *e.g.*, fibroblast cells, BHK cells, Vero cells, and CHO cells.

In particular embodiments, the methods of the invention may be carried out to achieve a higher yield of the polypeptide of interest. The polypeptide yield per cell may be increased by as much as about 2, 5, 10, 50, 100, 1000, 2500, 5000 or 10,000 fold or higher as compared with a suitable control cell (*e.g.*, in the absence of the inhibitory substance, the gene disruption, or the antisense sequence, and the like).

Moreover, the kinetics of polypeptide production may be altered so that higher levels of polypeptide production are observed at earlier time points, in particular in those aspects of the invention in which the cellular PKR pathway activity is inhibited or reduced.

The present invention may alternatively be carried out *in vivo* for the purposes of delivering a nucleic acid of interest to a subject, *e.g.*, for the purpose of antibody production, to produce an immune response, to produce a therapeutic effect (*e.g.*, to provide a protein that is deficient or defective in the subject, or to provide an otherwise therapeutic substance, such as an anti-cancer substance). Subjects, pharmaceutical formulations, and nucleic acids for achieving immunogenic or therapeutic effects are described in more detail below. The recombinant alphavirus genomic RNA may be delivered as an alphavirus particle or by another route, *e.g.*, a liposome formulation, as is known in the art.

The invention may be practiced to achieve higher levels of expression of the heterologous sequence *in vivo*, *e.g.*, by at least about 2, 5, 10, 100, 1000 or 10,000 fold as compared with expression in the a suitable control, *e.g.*, in the absence of the substance(s) that inhibits IFN responsiveness and/or PKR pathway activity. Moreover, the kinetics may be altered so that higher level expression of the heterologous nucleic acid may be observed at earlier time-points. Accordingly, the present invention may advantageously result in lower viral doses being administered to achieve the desired effects *in vivo*.

As another advantage, the present invention may be practiced to achieve viral infection and replication in cells that are normally non-permissive to alphavirus infection/replication, or only exhibit low levels thereof. For example, the present invention may be practiced to introduce an alphavirus vector (*e.g.*, a recombinant genomic alphavirus RNA or alphavirus particle) into antigen presenting cells, such as macrophages and dendritic cells. In particular, Sindbis virus (*e.g.*, TR339) vectors may be effectively delivered to macrophages and dendritic cells in the methods of the invention, whereas these cells normally restrict Sindbis virus infection/replication, presumably via IFN and/or PKR induced antiviral mechanisms.

Further, the present inventors have surprisingly found that the present invention may be practiced so as to enhance infection and/or replication within initially infected cells of interest, without altering the spread of virus to secondary (*i.e.*, surrounding or distal) cells. The invention may be practiced so as to substantially reduce the likelihood of propagation of the alphavirus beyond the initially infected cells as compared with the likelihood in the absence of the inventive methods and reagents.

This aspect of the present invention is desirable from a safety standpoint for *in vivo* applications, where it is advantageous to enhance infection of specific cells, such as antigen presenting cells (*e.g.*, macrophages and dendritic cells), but it is also desirable to control or limit the spread of virus to other cells in the subject. For example, according to this embodiment, an alphavirus replicon vector (*e.g.*, Sindbis), which is already propagation-incompetent, may be administered in the presence of a substance that

reduces cellular PKR pathway activity. According to this embodiment, a substance that otherwise inhibits cellular responsiveness to IFN is generally not administered to the subject.

While not wishing to be held to any particular theory of the invention, it appears that by specifically targeting the PKR-mediated antiviral response, while allowing alternative IFN-induced antiviral pathways to function, there is an enhanced level of alphavirus gene expression in initially infected cells, but virus spread by propagation-competent virus within the host is limited by alternative IFN signaling pathways. Therefore, the potential for propagation-competent virus to disseminate or cause disease in the host is substantially reduced. It is quite unexpected that the PKR pathway can be selectively inhibited to achieve higher levels of alphavirus gene expression in the initially infected cells, while leaving substantially intact the IFN-induced antiviral response in secondary cells.

VIII. Subjects, Pharmaceutical Formulations, Vaccines, and Modes of Administration.

The present invention finds use in both veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys and pheasants. The term "mammal" as used herein includes, but is not limited to, primates, bovines, ovines, caprines, porcines, equines, felines, canines, lagomorphs, rodents (*e.g.*, rats and mice), *etc.* Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile and adult subjects.

In particular embodiments, the present invention provides a pharmaceutical composition comprising an alphavirus vector of the invention in a pharmaceutically-acceptable carrier, and optionally containing other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid, such as sterile, pyrogen-free water or sterile pyrogen-free phosphate-buffered saline solution. For inhalation administration, the carrier will be respirable, and will preferably

be in solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer these reagents as an aerosol, or in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

The pharmaceutical composition may further comprise a substance that inhibits cellular responsiveness to IFN and or a substance that inhibits cellular PKR pathway activity, each as described hereinabove.

In other embodiments, the present invention provides a pharmaceutical composition comprising a cell that has been infected and genetically modified by an alphavirus vector in a pharmaceutically-acceptable carrier, and optionally containing other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, *etc.*

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, *e.g.*, the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example, in transfection of a cell *ex vivo* or in administering an alphavirus vector or cells directly to a subject.

Cells to be administered the inventive virus vectors *in vitro*, *ex vivo* or *in vivo*, can be of any type, including but not limited to antigen presenting cells (*e.g.*, macrophages, dendritic cells), neuronal cells (including cells of the peripheral and central nervous systems), retinal cells, epithelial cells (including dermal, gut, respiratory, bladder and breast tissue epithelium), muscle cells (including cardiac, smooth muscle, skeletal muscle, and diaphragm muscle), pancreatic cells (including islet cells), hepatic cells (*e.g.*, parenchyma), fibroblasts, endothelial cells, germ cells, lung cells (including bronchial cells and alveolar cells), prostate cells, stem cells, progenitor cells, dendritic cells, and the like. Alternatively, the cell is a cancer cell (including

tumor cells). Moreover, the cells can be from any species of origin, as indicated above.

Alternatively, in embodiments of the invention, the cell is a bone marrow cell or a cell in the bone-associated connective tissue. Other preferred cells, are cells of the periosteum, endosteum and tendons, generally within the epiphyses of the long bones adjacent to joints.

Cells that may be infected by the alphavirus vectors of the present invention further include, but are not limited to, polymorphonuclear cells, hemopoietic stem cells (including megakaryocyte colony forming units (CFU-M), spleen colony forming units (CFU-S), erythroid colony forming units (CFU-E), erythroid burst forming units (BFU-E), and colony forming units in culture (CFU-C)), erythrocytes, macrophages (including reticular cells), monocytes, granulocytes, megakaryoctyes, lymphocytes, fibroblasts, osteoprogenitor cells, osteoblasts, osteoclasts, marrow stromal cells, chondrocytes and other cells of synovial joints.

The alphavirus vectors of the invention may be administered to elicit an immunogenic response (*e.g.*, as an immunogenic composition or as a vaccine or for immunotherapy). Typically, immunological compositions of the present invention comprise an immunogenic amount of infectious virus particles as disclosed herein in combination with a pharmaceutically-acceptable carrier. An "immunogenic amount" is an amount of the infectious virus particles that is sufficient to evoke an active immune response in the subject to which the pharmaceutical formulation is administered. Typically, an amount of about 10^3 to about 10^{15} virus particles, about 10^4 to about 10^{13}, about 10^5 to about 10^{10}, or about 10^6 to 10^8 infectious virus particles per dose is suitable, depending upon the age and species of the subject being treated, and the immunogen against which the immune response is desired, as known by those skilled in the art. Subjects and immunogens are as described above. An alphavirus replicon particle is the preferred alphavirus vector.

The terms "vaccination" or "immunization" are well-understood in the art. For example, the terms vaccination or immunization can be understood to be a process that increases a subject's immune reaction to antigen and therefore the ability to resist or overcome infection. In the case of the present

invention, vaccination or immunization may also increase the organism's immune response and resistance to invasion by cancer or tumor cells.

Any suitable immunogenic composition and method of producing an immune response (*i.e.*, immunization) known in the art may be employed in carrying out the present invention, as long as an active immune response (preferably, a protective immune response) against the antigen is elicited. The immunogenic composition may further comprise a substance that inhibits cellular responsiveness IFN and/or a substance that reduced cellular PKR pathway activity, as described hereinabove.

An "active immune response" or "active immunity" is characterized by "participation of host tissues and cells after an encounter with the immunogen. It involves differentiation and proliferation of immunocompetent cells in lymphoreticular tissues, which lead to synthesis of antibody or the development of cell-mediated reactivity, or both." Herbert B. Herscowitz, *Immunophysiology: Cell Function and Cellular Interactions in Antibody Formation, in* IMMUNOLOGY: BASIC PROCESSES 117 (Joseph A. Bellanti ed., 1985). Alternatively stated, an active immune response is mounted by the host after exposure to immunogens by infection or by vaccination. Active immunity can be contrasted with passive immunity, which is acquired through the "transfer of preformed substances (antibody, transfer factor, thymic graft, interleukin-2) from an actively immunized host to a non-immune host." *Id.*

A "protective" immune response or "protective" immunity as used herein indicates that the immune response confers some benefit to the subject in that it prevents or reduces the incidence of disease. Alternatively, a protective immune response or protective immunity may be useful in the treatment of disease, in particular cancer or tumors (*e.g.*, by causing regression of a cancer or tumor and/or by preventing metastasis and/or by preventing growth of metastatic nodules). The protective effects may be complete or partial, as long as the benefits of the treatment outweigh any disadvantages thereof.

Administration of the immunogenic composition can be by any means known in the art, as described below, but is preferably by parenteral routes (*e.g.*, subcutaneous, intracerebral, intradermal, intramuscular, intravenous,

intraarticular), most preferably by subcutaneous injection. The dose of virus is not critical as long as it is sufficient to induce an active immune response to the expressed antigen.

The present invention further provides a method of delivering a nucleic acid to a cell (*e.g.*, to produce an immune response or for therapy). For *in vitro/ex vivo* methods, the virus may be administered to the cell by standard viral transduction methods, as are known in the art. Preferably, the virus particles are added to the cells at the appropriate multiplicity of infection according to standard transduction methods appropriate for the particular target cells. Titers of virus to administer can vary, depending upon the target cell type and the particular virus vector, and may be determined by those of skill in the art without undue experimentation.

In particular embodiments of the invention, cells are removed from a subject, the alphavirus vector is introduced therein, and the cells are then replaced back into the subject. Methods of removing cells from subject for treatment *ex vivo*, followed by introduction back into the subject are known in the art. Alternatively, the alphavirus vector is introduced into cells from another subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof. Preferably, if the subject's own cells are not used, the cells are HLA compatible with the subject's HLA type. The modified cell may be administered according to a method of *ex vivo* gene therapy or to provide immunity to a subject (*e.g.*, by introducing a nucleotide sequence encoding an immunogen into an antigen producing cell, such as a dendritic cell).

Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like, and may be routinely determined by those skilled in the art. Typically, at least about 10^2 to about 10^{10}, at least about 10^3 to about 10^8 cells, or about 10^4 to about 10^7 cells will be administered per dose. Preferably, the cells will be administered in a "immunogenic amount" (as described hereinabove) or a "therapeutically-effective amount".

A "therapeutically-effective" amount as used herein is an amount that is sufficient to alleviate (*e.g.*, mitigate, decrease, reduce) at least one of the symptoms associated with a disease state. Alternatively stated, a "therapeutically-effective" amount is an amount that is sufficient to provide some improvement in the condition of the subject.

A further aspect of the invention is a method of treating subjects *in vivo* with the inventive alphavirus particles to achieve therapeutic effects. Administration of the alphavirus particles of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors. The alphavirus delivery vector may be co-administered with a substance that inhibits cellular responsiveness to IFN and/or a substance that inhibits cellular PKR pathway activity, as described hereinabove (*e.g.*, in a single pharmaceutical formulation).

Dosages of the inventive alphavirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} infectious units or more, preferably about 10^8–10^{13} infectious units. Typically, an amount of about 10^3 to about 10^{15} virus particles, about 10^4 to about 10^{13}, about 10^5 to about 10^{10}, or about 10^6 to 10^8 infectious virus particles per dose is suitable.

Exemplary modes of administration to achieve for alphavirus vectors according to the present invention include oral, rectal, transmucosal, intranasal, topical, transdermal, inhalation, parenteral (*e.g.*, intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as well as direct tissue or organ injection, alternatively, intracerebral, intrathecal, direct intramuscular, intraventricular, intravenous, intraperitoneal, or intraocular injections. Alternatively, the alphavirus vector may be directly by implant or injection into or near a tumor.

In other preferred embodiments, the alphavirus vectors of the present invention are administered to the lungs. The alphavirus vectors disclosed herein may be administered to the lungs of a subject by any suitable means,

but are preferably administered by administering an aerosol suspension of respirable particles comprised of the inventive alphavirus vectors, which the subject inhales. The respirable particles may be liquid or solid. Aerosols of liquid particles comprising the inventive alphavirus vectors may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See, e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles comprising the inventive virus vectors may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Particular embodiments of the present invention are described in greater detail in the following non-limiting examples.

Example 1

Cell lines. Baby hamster kidney (BHK-21: ATCC CCL-10) and L929 murine fibrosarcoma cell lines were maintained in alpha minimal essential medium (αMEM), supplemented with 10% donor calf serum (DCS), 2.9 mg/ml tryptose phosphate, 0.29 mg/ml L-glutamine, 100 U/ml penicillin and 0.05 mg/ml streptomycin (37°C; 5% CO_2). NS47 murine fibroblasts were cultured in RPMI 1640, supplemented with 10% fetal calf serum, 0.29 mg/ml L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 20 μg/ml gentamicin, 5 mM Hepes buffer and 50 μM β-mercaptoethanol (cRPMI; Xu et al. (1995) *J. Immunol.* **154**: 2697-2705).

Example 2

Primary bone marrow-derived dendritic cells (BMDC). Bone-marrow cells from femurs and tibia of 8-12 week old mice were aspirated with cRPMI medium. On ice, cells were strained to remove bone fragments, pelleted (60*xg*; 5 min), counted and seeded at 5 x 10^7 cells per 150 mm diameter dish. Cells were incubated for 12-14 days (37°C, 5% CO_2) in cRPMI medium, supplemented with 5% NS47 fibroblast-conditioned culture supernatant and 500 ng/ml GM-CSF (Peprotech) (Xu et al. (1995) *J. Immunol.* **154**: 2697-2705). Every third day, dishes were gently swirled to dislodge non-adherent cells and two thirds of the supernatant was replaced with fresh,

supplemented cRPMI medium. Cells were harvested using enzyme-free cell-dissociation buffer (Gibco BRL) and gentle scraping, pelleted and seeded at 5 x 10^4 cells/cm^2 in 24-well and 60 mm plates in supplemented cRPMI medium. Prior to treatment or infection, cells were cultured a further two days.

Example 3

Sindbis viruses. Construction of the consensus sequence pTR339 plasmid has been described previously (Klimstra et al. (1998) *J. Virol.* **72**:7357-7366). Infectious viral RNA was generated by *in vitro* transcription using Message Machine kit (Ambion) from *Xho*I-linearized pTR339 DNA template and transfected into BHK-21 cells by electroporation. Virus particles were harvested from the supernatant 18-20 h after electroporation, clarified by centrifugation and stored at -70°C in single-use aliquots. Virus stocks were titered by standard BHK-21 cell plaque assay and titers were expressed as plaque forming units (PFU)/ml.

Example 4

Sindbis virus replicon particles. Sindbis virus replicon particles (SVRPs) were produced by packaging infectious replicon RNA transcripts in viral structural proteins provided in *trans* by helper RNAs (Bredenbeek et al. (1993) *J. Virol.* **67**:6439-6446). A replicon genome plasmid expressing green fluorescent protein (GFP) was constructed (p39REP-GFP), encoding the TR339 nonstructural protein genes and the *mut2 gfp* gene (kindly provided by Dr. Stanley Falkow, Stanford University) downstream of the 26S subgenomic promoter in place of the viral structural protein genes. The capsid protein helper (pCH) was similar to those described for other bipartite alphavirus replicon-packaging systems (Heise et al. (2000) *J. Virol.* **74**:9294-9299, Pushko et al. (1997) *Virol.* **239**:389-401). A glycoprotein helper plasmid (pINT) was engineered to produce a helper RNA from which the TR339 26S subgenomic promoter drives expression of a fusion protein consisting of the N-terminal 74 amino acids of TR339 capsid, a 2-amino acid linker, 17 amino acids of the foot and mouth disease virus (FMDV) 2A protease, and Sindbis virus PE2, 6K and E1 glycoproteins. RNA sequence from the N-terminal

truncated capsid forms a stem-loop structure that enhances translation of the fusion protein (Frolov et al. (1994) J. Virol. **68**:8111-8117, Frolov et al. (1996) *J. Virol.* **70**:1182-1190), while FMDV 2A protease cleaves at the FMDV 2A/PE2 junction allowing glycoprotein maturation, as shown previously with Semliki Forest virus (SFV)-derived (Smerdou, C. and P. Liljestrom. (1999) *J. Virol.* **73**:1092-1098) and S.A.AR86-derived (Heise et al. (2000) *J. Virol.* **74**:9294-9299) alphavirus replicons. *In vitro*-transcribed RNA from pINT, pCH, and p39REP-GFP were co-electroporated into BHK-21 cells and SVRPs were harvested 24 h post-electroporation. From each SVRP preparation, 10% was evaluated by serial passage on BHK-21 cells for the presence of cytopathic effect (cpe)-inducing, propagation-competent virus recombinants or contaminants (Macdonald, G. H. and R. E. Johnston. (2000) *J. Virol.* **74**:914-922). SVRP stocks were considered virus-free for experimental purposes if no cpe was evident after three serial amplifications. SVRP titers were determined by infection of BHK-21 cells and expressed as green infectious units (GIU)/ml.

Example 5

Mice. Breeder pairs of IFNAR1-/- and 129 Sv/Ev strain mice were kindly provided by Dr. Barbara Sherry (North Carolina State University, Raleigh, NC) and Dr. Herbert Virgin (Washington University, St. Louis, MS). Breeder pairs of PKR-/-, RNase L-/- and PKR/RNase L-/- (triply deficient [TD]) mice were kindly provided by Drs. Bryan Williams and Robert Silverman (Cleveland Clinic Foundation, Cleveland, OH). C57BL/6J strain mice were purchased from Jackson Laboratories. Mice were housed in the Department of Laboratory Animal Medicine animal facilities at the University of North Carolina at Chapel Hill under specific pathogen-free conditions. All procedures were carried out in accordance with institutional guidelines for animal care and use.

Example 6

Mortality studies. Virus inocula diluted in low-endotoxin, phosphate-buffered saline supplemented with 1% DCS (PBS-1% DCS) to contain 100 PFU TR339 in a 10 μl volume (1 x 10^4 PFU/ml) were administered

subcutaneously (s.c.) in each hind-leg footpad using a 30-gauge needle and Hamilton syringe. Mock-infected mice received 10 µl PBS-1% DCS by the same route. Virus-infected and corresponding mock-infected mice were observed at 12 h intervals, scored for degree of sickness, and weighed at 24 h intervals. Average survival times (AST) and percent mortality were calculated as described in Klimstra et al. (1999) *J. Virol.* **73**:10387-10398.

Example 7

Pathogenesis studies. At predetermined intervals post-infection (p.i.), groups of three mice per treatment were sacrificed under isoflurane-anesthesia (HSB Veterinary Supply) and blood was collected by cardiac puncture. Serum was separated from whole blood using microtainer tubes (Becton-Dickinson), aliquoted and stored at -70°C for assay of virus and IFNαβ. Each mouse was perfused with PBS-1% DCS for 10 min at 7 ml/min. The popliteal draining lymph nodes (DLNs), spleen and liver tissues were harvested into preweighed Kontes tubes and PBS-1% DCS was added to result in 10% suspensions. Tissues were homogenized by one freeze-thaw and mechanical disruption, and clarified by centrifugation (13,000 g, 15 min, 4°C). The supernatant was assayed for virus by standard plaque assay.

Example 8

Replicon inoculations and lymph node harvest. Under isoflurane anesthesia, 5×10^5 GIU of SVRP were inoculated s.c. in each rear footpad of two mice. Eight h p.i. mice were sacrificed and DLNs (popliteal) were harvested, immediately fixed in 4% paraformaldehyde (PFA, pH 7.4), embedded in OCT Compound (Tissue-Tek) and cryosectioned in 10-micron sections. SVRP-infected, GFP-expressing cells in 2-4 whole DLN were counted by fluorescence microscopy on a Nikon TE300 fluorescence microscope and recorded photographically at constant shutter speed to facilitate comparison of GFP fluorescence intensity between samples.

Example 9

***In vitro* virus replication.** For one-step growth curves, triplicate 2 cm^2 wells of BMDC were infected with TR339 at low (0.005 PFU/cell) or high (5 PFU/cell) multiplicity of infection (moi) in 200 µl. After 1 h infection at 37°C, cells were washed three times in PBS-1% DCS and medium replaced. In parallel experiments, triplicate wells of BMDC were pretreated with 10, 100 or 1000 international units (IU) IFNαβ (Access Biomolecular) or 10, 100 or 1000 effective dose (ED_{50})/ml IFNγ (R&D Systems) for 12 h prior to infection, or 5000 neutralizing units (NU) per well of anti-IFNαβ antiserum (Biosource) were added immediately following infection. At each time-point, 10 µl aliquots of BMDC supernatant from triplicate wells were independently titered by standard BHK plaque assay and titers were expressed as BHK PFU/ml.

Example 10

***In vitro* replicon infections and flow cytometry.** Triplicate 2 cm^2 wells of BMDC, either untreated or pretreated for 12 h with IFNαβ or IFNγ (as described above), were infected with SVRPs at high moi (50 GIU/cell). Immediately following infection, 5000 NU anti-IFNαβ antiserum were added to some wells. Expression of GFP was monitored by fluorescence microscopy and recorded photographically at constant shutter speed to facilitate comparison of GFP fluorescence intensity between samples. In parallel experiments, SVRP-infected and uninfected BMDC were treated with enzyme-free cell-dissociation buffer at 37°C for 5 min and harvested with a sterile cell-scraper. Cells were pelleted in V-bottom wells and resuspended in PBS-500 µM EDTA at approx. 5 x 10^5 cells/ml. The mean fluorescence intensity of GFP-positive infected cells was quantitated by flow cytometric analysis on a FACScan II flow cytometer (Flow Cytometry Facility, University of North Carolina).

Example 11

IFNαβ biological assays. Serum IFNαβ was titered by standard biological assay on L929 cells as described previously (Trgovcich et al., (1996) *Virology* **224**:73), using a commercially prepared IFNαβ standard

(Access Biomolecular) and EMCV as the indicator virus. Endpoint was defined as the dilution of IFNαβ required to protect 50% of the cells from EMCV-induced cpe and the level of IFNαβ was expressed as IU/ml or g.

Example 12

Statistical analyses. The statistical significance of the data was determined by two-tailed Student *t*-test.

Example 13

Sindbis virus virulence in the absence of PKR and/or RNase L. Our previous studies have demonstrated that the survival of adult mice is dependent upon IFNαβ signaling through the IFNAR receptor following infection with consensus wild-type Sindbis virus TR339 (Ryman et al. (2000) *J. Virol.* **74**:3366-3378). To determine the relative contributions of the major IFNαβ-mediated antiviral pathways to protection of mice from fatal Sindbis virus infection, the virulence of TR339 was compared in PKR-/- (Yang et al. (1995) *EMBO J.* **14**:6095-6106), RNase L-/- (88), TD (PKR/RNase L-/-; 89), IFNAR1-/- (47), 129 Sv/Ev and C57BL/6J (Jackson) mice. All of these mouse strains are deficient in Mx1 protein activity (Staeheli et al. (1988) *Mol. Cell. Biol.* **8**:4518-4523, Zhou et al. (1999) *Virol.* **258**:435-440). Groups of four female 6-7 week-old mice were inoculated with 100 PFU of TR339 s.c. in each rear footpad, clinically scored at 12 h intervals and weighed daily (**Table 2**). Confirming our previous observations, IFNAR1-/- mice were extremely susceptible to TR339 infection (100% mortality), succumbing 63.0 ± 0.6 h p.i. In contrast, PKR-/-, RNase L-/- and TD mice appeared no more susceptible to disease than C57BL/6J or 129 Sv/Ev controls. No morbidity was observed. Increasing the virus inoculum dose to 1 x 10^5 PFU or inoculating intracerebrally shortened the AST of infected IFNAR1-/- mice, but had no clinical effect on the susceptibility of the other mouse genotypes.

	Morbidity[b]	Mortality[c]	AST +/- SD (Hours)
129 Sv/Ev	0%	0%	N/A[d]
C57BL/6J	0%	0%	N/A
IFNAR1-/-	100%	100%	63.0 +/- 0.6
PKR-/-	0%	0%	N/A
RL-/-	0%	0%	N/A
TD	0%	0%	N/A

Table 2: Sindbis virus virulence in the absence of PKR and/or RNase L. [a]Mice were inoculated s.c. with 100 PFU TR339 virus. The data represented in this table were collected in one experiment. However, the results are representative of two independent experiments. No morbidity or mortality was observed in any TR339-infected 129 Sv/Ev, C57BL/6, TD, PKR-/- or RNase L-/- mice. [b]Morbidity is defined by > 10% weight loss, ruffling of fur, ataxia, paresis or paralysis. [c]Percent mortality during a 3-week observation period following inoculation. [d]N/A, not applicable.

Example 14

Effects of PKR, RNase L and alternative antiviral pathways on virus particle production *in vivo*. When injected s.c., alphaviruses infect macrophages and DC at the inoculation site that subsequently migrate to the DLN (Gardner et al. (2000) *J. Virol.* **74**:11849-11857, Macdonald, G. H. and R. E. Johnston. (2000) *J. Virol.* **74**:914-922, Ryman et al. (2000) *J. Virol.* **74**:3366-3378), where the virus continues to replicate. In the absence of IFNαβ signaling through the IFNAR receptor, these cells become much more permissive to Sindbis virus infection and a high titer viremia is seeded. Resident tissue DCs and macrophages become infected systemically, resulting in a fatal SIRS-like proinflammatory cytokine cascade (Ryman et al. (2000) *J. Virol.* **74**:3366-3378). To investigate the combined effect of PKR and RNase L pathways on the early stages of virus replication and dissemination *in vivo*, TD, IFNAR1-/- and 129 Sv/Ev mice inoculated s.c. with 100 PFU TR339 were sacrificed at 4, 8, 12, and 24 h p.i. and the virus content of serum and PBS-perfused DLN, spleen and liver were determined by plaque assay (**Figure 1, Panels A** and **B**). At 4 h p.i. the viral load in DLN of TD mice was

significantly greater than in either 129 Sv/Ev or IFNAR1-/- DLN (**Figure 1, Panel A**). However, by 24 h p.i. more extensive replication was detected in IFNAR1-/- DLN. At the earliest time virus replication was detectable beyond the DLN (in serum, spleen and liver), levels were highest in the tissues of IFNAR1-/- mice (**Figure 1, Panel B**). Compared to TD mice, virus replication and dissemination were slightly suppressed by PKR/RNase L-mediated mechanisms in 129 Sv/Ev tissues.

Example 15

Targeting of SVRPs *in vivo*. SVRPs outwardly resemble the parental virus, but are able to undergo only a single round of replication (reviewed in Schlesinger et al., (1999) *Curr. Opin. Biotechnol.* **10**:434). Thus, SVRPs expressing GFP were used to identify and enumerate the initial cellular targets of viral replication in mice lacking PKR/RNase L or the IFNαβ receptor. Examination of DLN sections from SVRP-inoculated mice 8 h p.i. revealed GFP-positive cells of macrophage/DC morphology concentrated primarily beneath the capsule on the afferent side of the LN (**Figure 2, Panels A, B,** and **C**), as described previously (Macdonald, G. H. and R. E. Johnston. (2000) *J. Virol.* **74**:914-922, Ryman et al. (2000) *J. Virol.* **74**:3366-3378). GFP-positive cells were observed relatively rarely in DLN sections from SVRP-inoculated 129 Sv/Ev mice (432 cells/DLN where *n=2*; **Figure 2 Panel A**). In contrast, GFP-positive cells were abundant in DLN harvested from both IFNAR1-/- (1959 cells/DLN where *n=4*; **Figure 2 Panel B**) and TD mice (2992 cells/DLN where *n=4*; **Figure 2 Panel C**).

Example 16

Effects of PKR, RNase L and alternative antiviral pathways on virus replication in primary BMDC. Preliminary experiments performed in immortalized murine embryo fibroblast (MEF) cell lines derived from PKR-/- and RNase L-/- mice or their respective controls (PKR+/+ and RNase L+/+) suggested that both PKR and RNase L contributed to the IFNαβ-induced anti-Sindbis virus response (data not shown). However, inherent problems were encountered with these assays as the passage history of the control cell lines

dramatically influenced their permissivity to Sindbis virus infection, such that the PKR+/+ cells were significantly more permissive than the RNase L+/+ cells.

$CD11b^+$, $CD11c^+$ macrophage/DC lineage cells were previously identified as targets for Sindbis virus replication *in vivo* (Gardner et al. (2000) *J. Virol.* **74**:11849-11857, Ryman et al. (2000) *J. Virol.* **74**:3366-3378). Thus, by generating primary BMDC from the genetically-modified mice, it was possible to characterize the effects of IFN signaling and IFN-mediated antiviral pathways on Sindbis virus replication in a cell-type relevant to alphavirus pathogenesis. Maintenance of BM cells in GM-CSF and NS47 fibroblast-conditioned supernatant selected for the growth of immature, myeloid-lineage DC expressing cell-surface CD11b, CD11c and DEC205, similar to previously described XS52 and XS106 immature DC cell lines (Xu et al. (1995) *J. Immunol.* **154**: 2697-2705, Xu et al. (1995) *Eur. J. Immunol.* **25**: 1018-1024). Primary BMDC derived from TD, IFNAR1-/-, and 129 Sv/Ev mice were infected with TR339 virus at low or high moi and progeny virions released into the cell supernatant were quantitated (**Figure 3 Panels A** and **B**). In a low moi infection (moi = 0.005 PFU/cell), a small subset of cells is infected initially, such that the effects of IFN pathway components on both virus replication and spread within the culture are evident. Low levels of virus were released from background 129 Sv/Ev BMDC, whereas both TD and IFNAR1-/- derived BMDC were significantly more permissive in terms of virion production (**Figure 3 Panel A**). The release of progeny virions from TD-derived cells was detectable 6-12 h earlier than from IFNAR1-/- or 129 Sv/Ev BMDC. This was suggestive that, similar to *in vivo* observations, the PKR/RNase L-/- pathways significantly suppress early virus replication, even in the absence of IFNαβ signaling. However, by 30 h p.i. 100-fold more virus had been produced by IFNAR1-/- than TD cells indicative of an autocrine or priming IFNαβ activity absent in cells lacking the IFNAR1 receptor subunit.

By infecting at high moi (moi = 5 PFU/cell), virtually every cell was exposed to multiple infectious virus particles during the initial infection period and consequently the virus titer assayed from the supernatant represented the progeny virions from a synchronized infection of these cells (**Figure 3**

Panel B). Significantly more virions were released from TD-derived than from IFNAR1-/- derived BMDC during the early stages of the infection (6 through 9 h p.i.). However, cumulative virion production from IFNAR1-/- cells equaled (12 h p.i.) and then exceeded production from TD cells later in infection. Again, these data suggest that the early events in virus replication and/or morphogenesis were inhibited by the presence of PKR and/or RNase L in the infected cell in the absence of IFNαβ signaling. By 12 h p.i., however, a non-PKR/non-RNase L autocrine pathway, mediated by signaling through the IFNAR receptor, began to act on the infected cell to suppress virus replication and/or release. Thus, signaling of induced IFNαβ through the IFNAR receptor in infected TD-derived cells appeared to significantly suppress virus replication and/or egress.

In order to determine the relative contributions of PKR and RNase L to the early antiviral effect observed in TD-derived cells, virus growth curves were also performed in BMDC derived from PKR-/-, RNase L-/- and C57BL/6J mice (**Figure 3 Panel C**). Comparison of virus replication following high moi infection indicated that significantly more virions were produced from PKR-/- BMDC than either RNase L-/- or C57BL/6 BMDC. Thus, it appeared that the PKR pathway was primarily responsible for the early suppression of virus replication observed in the 129 Sv/Ev and IFNAR1-/- BMDC.

Example 17

Antiviral activity of induced IFNαβ in the absence of PKR/RNase L pathways. To determine whether or not virus replication in TD cultures was limited by an autocrine and/or paracrine activity of induced IFNαβ, TD- and 129 Sv/Ev-derived BMDC were infected in the presence of neutralizing anti-IFNαβ antiserum (**Figure 4**). Neutralization of induced IFNαβ significantly enhanced early replication in TD cells and slowed the suppressive effects later in infection. The levels of progeny virions reached were not significantly different from those attained in IFNAR1-/- cells ($p > 0.2$). This was suggestive that exogenous IFNαβ, induced by virus infection of BMDC, could signal through the IFNAR receptor to exert an antiviral effect in the absence of PKR and RNase L.

Viral infection of BMDC derived from IFNAR1-/- mice resulted in cpe by 30 h p.i. (**Figure 5 Panel A**). Notably, little or no evidence of cpe was observed in infected cultures of 129 Sv/Ev or TD-derived cells (**Figures 5 Panels B** and **C**, respectively). In the case of 129 Sv/Ev BMDC, the most likely explanation for this finding was that the virus did not replicate sufficiently well to cause cpe. However, virus replication in the TD-derived BMDC was extensive and infections with a replication-competent, double-promoter Sindbis virus expressing GFP suggested that all the cells were permissive to infection (data not shown). When neutralizing anti-IFNαβ antibodies were added to the TD cultures, cpe occurred, suggestive that IFNαβ signaling was required for protection of infected cells from cpe (**Figure 5 Panel D**).

Example 18

Replicon GFP-expression in BMDC. BMDC were infected at high moi with GFP-expressing SVRP as an indicator of productive infection and the level of protein expression from the viral subgenomic promoter (**Figure 6 Panels A-D**). Whereas GFP fluorescence was barely discernable in BMDC derived from 129 Sv/Ev or IFNAR1-/- mice (**Figures 6 Panels A** and **B**, respectively), high level expression of GFP was observed in TD-derived cells (**Figure 6 Panel C**). When quantitated, the mean fluorescence intensity of GFP 8 h p.i. in TD-derived BMDC was approximately 10-fold higher than the mean fluorescence intensity in either IFNAR1-/- or 129 Sv/Ev-derived cells (**Figure 7**), suggesting that enhanced viral gene expression within individual TD cells accounted for the increased titers observed in the growth curves. As described for the virus growth curves, enhanced expression of GFP from the subgenomic promoter cosegregated with the ablation of PKR, such that GFP fluorescence in PKR-/- BMDC was over 10-fold higher than in RNase L-/- or C57BL/6 cells. Taken together, these *in vitro* data suggested that the antiviral effects of constitutively expressed or rapidly induced PKR dramatically suppress virus replication in normal cells.

Example 19

Induction of IFNαβ *in vivo* and *in vitro* in the absence of PKR/RNase L pathways. As described above, virus titers and GFP expression in TD-derived BMDC were suppressed later in the infection. Likewise, DLN titers in TD mice were suppressed later in infection and virus systemic replication was limited. This could be accounted for by uninhibited early virus replication leading to hyper-induction of IFNαβ and suppression of late replication by a physiologically-irrelevant mechanism. To test this hypothesis levels of IFNαβ induced by virus infection of TD, 129 Sv/Ev or IFNAR1-/- mice or BMDC cultures were measured (**Figure 8 Panels A** and **B**).

In vivo, IFNαβ induction was not detectable (≤ 20 IU/ml) 4 h p.i., but was detectably induced in TD and 129 Sv/Ev mice by 8 h p.i., increasing through 12 h p.i. and falling below the limit of detection by 24 h p.i. (**Figure 8 Panel A**). The level of induction was 5-fold higher in TD compared to 129 Sv/Ev animals. By 24 h p.i. the virus infection had largely been cleared and IFNαβ release was reduced. In contrast, detectable induction of IFNαβ was delayed in infected IFNAR1-/- mice until 12 h p.i., then increased to very high levels by 24 h p.i.

The extent of IFNαβ induction and release from BMDC following virus infection was also determined (**Figure 8 Panel B**). The absence of PKR/RNase L significantly delayed and suppressed IFNαβ induction in comparison with 129 Sv/Ev control cells. This result may be explained by a more rapid virus-induced shutoff of host protein synthesis with earlier virus replication in the absence of PKR/RNase L. Alternatively, a role for activated PKR in dsRNA-triggered IFNαβ induction may be indicated, as suggested previously (Chu et al. (1999) *Immunity* **11**:721-731, Khabar et al. (2000) *J. Interferon Cytokine Res.* **20**:653-659). As observed *in vivo*, in the absence of IFNαβ receptor-mediated signaling, the induction and release of IFNαβ was also reduced. In IFNAR1-/- cells, the IFNαβ response cannot be amplified through receptor-mediated signaling and likely is restricted to IFNβ and IFNα4 release. The *in vitro* and *in vivo* data described above suggested that comparable levels of IFNαβ induced by virus infection of BMDC suppressed

virus replication later in infection regardless of the presence or absence of PKR/RNase L.

Example 20

IFNαβ and IFNγ priming in the absence of PKR/RNase L pathways.

To further investigate the effects of exogenous IFNαβ or IFNγ priming on virus replication, BMDC were pretreated for 12 h with 10, 100 or 1000 IU/ml IFNαβ or ED_{50}/ml IFNγ prior to infection with TR339 virus at high moi (**Figure 9 Panels A-F**). The production and release of progeny virions from infected 129 Sv/Ev-derived BMDC was almost completely prevented at the lowest dose of IFNαβ (10 IU) or IFNγ (10 ED_{50}). This antiviral effect remained in effect through 30 h p.i. although the IFN was removed at the time of infection. As expected, IFNαβ had no significant antiviral effect on IFNAR1-/- BMDC. Interestingly, although IFNγ was able to suppress virion production in IFNAR1-/- cells through 6 h p.i., approximately one half of the antiviral effect was lost by 12 h p.i. and did not appear to be dose-dependent. A significant residual IFNαβ-mediated antiviral effect was observed in TD-derived BMDC. The efficacy and longevity of this effect was dose-dependent, such that by 30 h p.i. only the highest dose (1000 IU) continued to completely suppress virus release below the level of detection. In comparison with IFNαβ-mediated antiviral effect in 129 Sv/Ev-derived cells, approximately 10-fold more IFNαβ was required for a similar effect. IFNγ completely suppressed virion production and release from TD-derived cells even at the lowest dose (10 ED_{50}). Recombinant murine IFNα-2a and IFNβ (Calbiochem) appeared to be equally effective at inducing an antiviral state in the BMDC (data not shown).

Priming with 1000 IU IFNαβ prior to SVRP infection greatly reduced the mean fluorescence intensity of GFP expression in TD- and PKR-/- derived BMDC (**Figure 10**). IFNγ priming was equally effective on TD-derived BMDC. As GFP is a cumulative indicator of translation from the subgenomic promoter, these data suggest that the induction of ISGs within the primed cell inhibited early steps in the virus replication cycle either at the level of transcription or translation.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A method of producing a recombinant alphavirus particle *in vitro*, comprising:

providing to a cell

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence;

(b) a sequence(s) encoding the alphavirus structural proteins sufficient for producing an alphavirus particle; and

(c) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity;

wherein the combined expression of the recombinant alphavirus genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

2. A method of producing a recombinant alphavirus particle, comprising:

providing to a cell having a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence; and

(b) a sequence(s) encoding the alphavirus proteins sufficient to produce an alphavirus particle;

wherein the combined expression of the recombinant alphavirus genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

3. The method of Claim 2, wherein the cell does not have detectable PKR activity.

4. The method of Claim 1, wherein an RNA sequence that encodes the substance that inhibits cellular PKR pathway activity is provided to the cell.

5 The method of Claim 4, wherein the sequence(s) encoding the alphavirus structural proteins further comprise the RNA sequence that encodes the substance that inhibits cellular PKR pathway activity.

6. The method of Claim 4, wherein a recombinant alphavirus genomic RNA comprises the RNA sequence that encodes the substance that inhibits cellular PKR pathway activity is provided to the cell.

7. The method of any of Claims 4 to 6, wherein the RNA sequence is expressed from a DNA sequence in the cell.

8. The method of Claim 7, wherein the DNA sequence is stably expressed by the cell by integration into the cellular DNA or from an episome.

9. The method of Claim 1, wherein the substance that inhibits cellular PKR pathway activity is selected from the group consisting of 2-aminopurine, adenovirus VAI RNA, influenza A Ns1 protein, vaccinia E3L protein, Herpes simplex virus ICP34.5 protein, p58ipk protein, an autologous dominant negative PKR inhibitor, an antibody, an antisense nucleic acid, and an interfering RNA.

10. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are expressed from a DNA sequence in the cell.

11. The method of Claim 10, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are stably expressed by the cell by integration into the cellular DNA or from an episome.

12. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are provided to the cell as RNA molecules.

13. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA comprises sequence(s) encoding at least one of the alphavirus structural proteins.

14. The method of Claim 13, wherein the recombinant alphavirus genomic RNA comprises the sequence(s) encoding the alphavirus structural proteins sufficient to produce an alphavirus particle.

15. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA is propagation-defective in that it does not encode one or more of the alphavirus structural proteins

16. The method of Claim 15, wherein the recombinant alphavirus genomic RNA lacks the sequences encoding the alphavirus structural proteins.

17. The method of Claim 1 or 2, wherein the heterologous RNA sequence is operatively associated with a promoter.

18. The method of Claim 17, wherein the heterologous RNA sequence is operatively associated with an alphavirus 26S subgenomic promoter.

19. The method of Claim 1 or 2, wherein the heterologous RNA sequence encodes a polypeptide.

20. The method of Claim 19, wherein the heterologous RNA sequence encodes an immunogenic polypeptide.

21. The method of Claim 20, wherein the heterologous RNA sequence encodes an immunogenic polypeptide from a pathogenic organism or virus, a cancer cell, or a tumor cell.

22. The method of Claim 19, wherein the heterologous RNA sequence encodes a therapeutic polypeptide.

23. The method of Claim 1 or 2, wherein the heterologous RNA sequence encodes an antisense sequence, an interfering RNA, or other untranslated RNA molecule.

24. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, comprise or encode an attenuating mutation.

25. The method of Claim 1 or 2, wherein the recombinant alphavirus genomic RNA is selected from the group consisting of a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, Sindbis genomic RNA, S.A.AR86 genomic RNA, and Semliki Forest Virus genomic RNA.

26. The method of Claim 1 or 2, wherein the sequence(s) encoding the alphavirus structural proteins are selected from the group consisting of a sequence(s) encoding Venezuelan Equine Encephalitis (VEE), Sindbis, and Semliki Forest Virus structural proteins.

27. The method of Claim 25, wherein the alphavirus genomic RNA is a TR339 virus genomic RNA.

28. The method of Claim 26, wherein the sequence(s) encoding the alphavirus structural proteins encode TR339 structural proteins.

29. The method of Claim 1 or 2, wherein the number of alphavirus particles produced is at least ten-fold greater as compared with a control cell.

30. The method of Claim 1 or 2, wherein the cell is selected from the group consisting of a Baby Hamster Kidney cell, a Chinese Hamster Ovary cell, a DF-1 cell, a Vero cell, a 293 cell, and a 293T cell.

31. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence; and

(b) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity;

wherein the heterologous RNA sequence is introduced into and expressed in the cell.

32. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell having a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity:

a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence;

wherein the heterologous RNA sequence is expressed in the cell.

33. The method of Claim 31 or 32, wherein the number of alphavirus particles produced is at least ten-fold greater as compared with a control cell.

34. The method of Claim 31 or 32, wherein the cell is a dendritic cell.

35. The method of Claim 31 or 32, wherein the cell is a cancer or tumor cell.

36. The method of Claim 31 or 32, wherein the cell is a cell *in vitro*.

37. The method of Claim 36, further comprising the step of administering the cell to a subject.

38. The method of Claim 31, wherein the cell is a cell *in vivo* in a subject.

39. The method of Claim 38, wherein the recombinant alphavirus genomic RNA is administered to the subject by a route selected from the group consisting of intradermal administration, intravenous administration, subcutaneous administration, intramuscular administration, intranasal administration, administration to the lungs, and direct administration by implant or injection into a tissue or tumor.

40. The method of Claim 38, wherein the subject is selected from the group consisting of a human, simian, equine, rat, mouse, lagamorph, porcine, caprine, ovine, bovine, canine, feline and avian subject.

41. The method of Claim 31 or 32, wherein an alphavirus particle comprising the recombinant alphavirus genome is contacted with the cell.

42. A recombinant alphavirus genomic RNA comprising a heterologous RNA sequence and a sequence that encodes a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity.

43. An alphavirus particle comprising the recombinant alphavirus genomic RNA of Claim 42.

44. A pharmaceutical formulation comprising the recombinant alphavirus genomic RNA of Claim 42 or the alphavirus particle of Claim 43 in a pharmaceutically acceptable carrier.

45. A DNA molecule encoding the recombinant alphavirus genomic RNA of Claim 42.

46. A cell comprising the recombinant alphavirus genomic RNA of Claim 42 or the DNA molecule of Claim 45.

47. A helper cell for producing an alphavirus particle, comprising:
(a) a sequence encoding an alphavirus structural protein; and
(b) a sequence encoding a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity.

48. The method of Claim 47, wherein the sequences of (a) and (b) are DNA sequences and are stably expressed by the cell by integration into the cellular DNA or from an episome.

49. A helper cell for producing an alphavirus particle, comprising: a cell having essentially no detectable double-stranded RNA dependent protein kinase (PKR) activity, said cell comprising a sequence encoding an alphavirus structural protein.

50. The helper cell of Claim 49, wherein the sequence encoding the alphavirus structural protein is a DNA sequence and is stably expressed by the cell by integration into the cellular DNA or from an episome.

51. The helper cell of any of Claims 47 to 50, wherein the cell further comprises a sequence(s) encoding the alphavirus structural proteins sufficient to produce an alphavirus particle.

52. The helper cell of any of Claims 47 to 51, wherein the cell further comprises an alphavirus replicon RNA.

53. An isolated nucleic acid comprising (i) a sequence encoding at least one alphavirus structural protein, and (ii) a sequence encoding a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity in a cell.

54. A method of producing a recombinant alphavirus particle *in vitro*, comprising:

providing to a cell

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence;

(b) a sequence(s) encoding the alphavirus structural proteins sufficient for producing an alphavirus particle;

(c) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity; and

(d) a substance that inhibits cellular responsiveness to interferon;

wherein the combined expression of the recombinant alphavirus genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

55. A method of producing a recombinant alphavirus particle, comprising:

providing to a cell having a reduced responsiveness to interferon, and optionally a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity:

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence; and

(b) a sequence(s) encoding the alphavirus proteins sufficient to produce an alphavirus particle;

wherein the combined expression of the recombinant alphavirus genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant alphavirus genomic RNA comprising the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

56. The method of Claim 55, wherein the cell lacks detectable interferon α/β (IFNAR) receptors.

57. The method of Claim 55, wherein the cell lacks detectable interferon receptors.

58. The method of Claim 55, wherein the cell is selected from the group consisting of a Baby Hamster Kidney cell, a Chinese Hamster Ovary cell, a DF-1 cell, a Vero cell, a 293 cell, and a 293T cell.

59. The method of Claim 55, wherein the cell does not have detectable PKR activity.

60. The method of Claim 55 wherein the cell has a reduced cellular responsiveness to interferon and further comprising providing to the cell a substance that inhibits cellular PKR pathway activity.

61. The method of Claim 55, wherein the cell has reduced PKR pathway activity and further comprising providing to the cell a substance that inhibits cellular responsiveness to interferon.

62. The method of Claim 54 or Claim 60, wherein an RNA sequence that encodes the substance that inhibits cellular PKR pathway activity is provided to the cell.

63. The method of Claim 62 wherein the sequence(s) encoding the alphavirus structural proteins further comprise the RNA sequence that encodes the substance that inhibits cellular PKR pathway activity.

64. The method of Claim 62 wherein a recombinant alphavirus genomic RNA comprises the RNA sequence that encodes the substance that inhibits cellular PKR pathway activity is provided to the cell.

65. The method of any of Claims 62 to 64, wherein the RNA sequence is expressed from a DNA sequence in the cell.

66. The method of Claim 65, wherein the DNA sequence is stably expressed by the cell by integration into the cellular DNA or from an episome.

67. The method of Claim 54 or 60, wherein the substance that inhibits cellular PKR pathway activity is selected from the group consisting of 2-aminopurine, adenovirus VAI RNA, influenza A Ns1 protein, vaccinia E3L protein, Herpes simplex virus ICP34.5 protein, p58ipk protein, an autologous dominant negative PKR inhibitor, an antibody, an antisense nucleic acid, and an interfering RNA.

68. The method of Claim 54 or Claim 61, wherein an RNA sequence that encodes the substance that inhibits cellular responsiveness to interferon is provided to the cell.

69. The method of Claim 68, wherein the sequence(s) encoding the alphavirus structural proteins comprise the RNA sequence that encodes the substance that inhibits cellular responsiveness to interferon.

70. The method of Claim 68, wherein a recombinant alphavirus genomic RNA comprises the RNA sequence that encodes the substance that inhibits cellular responsiveness to PKR is provided to the cell.

71. The method of any of Claims 68 to 70, wherein the RNA sequence is expressed from a DNA sequence in the cell.

72. The method of Claim 71, wherein the DNA sequence is stably expressed by the cell by integration into the cellular DNA or from an episome.

73. The method of Claim 54 or 61, wherein the substance that inhibits cellular responsiveness to interferon is selected from the group consisting of Ebola VP35 protein, bunyavirus NS protein, Sendai C protein, simian virus 5 V protein, vaccinia virus VH1 protein, an antibody, an interferon receptor antagonist, an antisense nucleic acid, and an interfering RNA.

74. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are expressed from a DNA sequence in the cell.

75. The method of Claim 74, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are stably expressed by the cell by integration into the cellular DNA or from an episome.

76. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, are provided to the cell as RNA molecules.

77. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA comprises a sequence(s) encoding at least one of the alphavirus structural proteins.

78. The method of Claim 77, wherein the recombinant alphavirus genomic RNA comprises the sequence(s) encoding the alphavirus structural proteins sufficient to produce an alphavirus particle.

79. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA is propagation-defective in that it does not encode one or more of the alphavirus structural proteins

80. The method of Claim 79, wherein the recombinant alphavirus genomic RNA lacks the sequence(s) encoding the alphavirus structural proteins.

81. The method of Claim 54 or 55, wherein the heterologous RNA sequence is operatively associated with a promoter.

82. The method of Claim 81, wherein the heterologous RNA sequence is operatively associated with an alphavirus 26S subgenomic promoter.

83. The method of Claim 54 or 55, wherein the heterologous RNA sequence encodes a polypeptide.

84. The method of Claim 83, wherein the heterologous RNA sequence encodes an immunogenic polypeptide.

85. The method of Claim 84, wherein the heterologous RNA sequence encodes an immunogenic polypeptide from a pathogenic organism or virus, a cancer cell, or a tumor cell.

86. The method of Claim 83, wherein the heterologous RNA sequence encodes a therapeutic polypeptide.

87. The method of Claim 54 or 55, wherein the heterologous RNA sequence encodes an antisense sequence, an interfering RNA, or other untranslated RNA molecule.

88. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA or the sequence(s) encoding the alphavirus structural proteins, or both, comprise or encode an attenuating mutation.

89. The method of Claim 54 or 55, wherein the recombinant alphavirus genomic RNA is selected from the group consisting of a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, Sindbis genomic RNA, S.A.AR86 genomic RNA, and Semliki Forest Virus genomic RNA.

90. The method of Claim 54 or 55, wherein the sequence(s) encoding the alphavirus structural proteins are selected from the group consisting of sequence(s) encoding Venezuelan Equine Encephalitis (VEE), Sindbis, and Semliki Forest Virus structural proteins.

91. The method of Claim 89, wherein the alphavirus genomic RNA is a TR339 virus genomic RNA.

92. The method of Claim 90, wherein the sequence(s) encoding the alphavirus structural proteins encode TR339 structural proteins.

93. The method of Claim 89, wherein the alphavirus genomic RNA is a VEE virus genomic RNA comprising a mutation at nucleotide 3 of the VEE genomic RNA.

94. The method of Claim 93, wherein the mutation at nucleotide 3 is a G→A mutation.

95. The method of Claim 54 or 55 wherein the number of alphavirus particles produced is at least ten-fold greater as compared with a control cell.

96. The method of Claim 54 or 55, wherein the is selected from the group consisting of a Baby Hamster Kidney cell, a Chinese Hamster Ovary cell, a DF-1 cell, a Vero cell, a 293 cell, and a 293T cell.

97. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell

(a) a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence;

(b) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity; and

(c) a substance that inhibits cellular responsiveness to interferon;

wherein the heterologous RNA sequence is introduced into and expressed in the cell.

98. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell having a reduced responsiveness to interferon, and optionally a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity:

a recombinant alphavirus genomic RNA comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence;

wherein the heterologous RNA sequence is expressed in the cell.

99. The method of Claim 98, wherein the cell has reduced responsiveness to interferon and further comprising providing to the cell a substance that inhibits cellular PKR pathway activity.

100. The method of Claim 98, wherein the cell has reduced PKR pathway activity and further comprising providing to the cell a substance that inhibits cellular responsiveness to interferon.

101. The method of Claim 97 or 98, wherein the number of alphavirus particles produced is at least ten-fold greater as compared with a control cell.

102. The method of Claim 97 or 98, wherein the cell is a dendritic cell.

103. The method of Claim 97 or 98, wherein the cell is a cancer or tumor cell.

104. The method of Claim 97 or 98, wherein the cell is a cell *in vitro*.

105. The method of Claim 104, further comprising the step of administering the cell to a subject.

106. The method of Claim 97, wherein the cell is a cell *in vivo* in a subject.

107. The method of Claim 106, wherein the recombinant alphavirus genomic RNA is administered to the subject by a route selected from the group consisting of intradermal administration, intravenous administration, subcutaneous administration, intramuscular administration, intranasal

administration, administration to the lungs, and direct administration by implant or injection into a tissue or tumor.

108. The method of Claim 106, wherein the subject is selected from the group consisting of a human, simian, equine, rat, mouse, lagamorph, porcine, caprine, ovine, bovine, canine, feline and avian subject.

109. The method of Claim 97 or 98, wherein an alphavirus particle comprising the recombinant alphavirus genome is contacted with the cell.

110. A recombinant alphavirus genomic RNA comprising a heterologous RNA sequence and a sequence that encodes a substance that inhibits cellular responsiveness to interferon.

111. The method of Claim 110, wherein the recombinant alphavirus genomic RNA further comprises a sequence that encodes a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity.

112. An alphavirus particle comprising the recombinant alphavirus genomic RNA of Claim 110 or 111.

113. A pharmaceutical formulation comprising the recombinant alphavirus genomic RNA of Claim 110 or 111 or the alphavirus particle of Claim 58 in a pharmaceutically acceptable carrier.

114. A DNA molecule encoding the recombinant alphavirus genomic RNA of Claim 110 or 111.

115. A cell comprising the recombinant alphavirus genomic RNA of Claim 110 or 111 or the DNA molecule of Claim 60.

116. A helper cell for producing an alphavirus particle, comprising:
(a) a sequence encoding an alphavirus structural protein;
(b) a sequence encoding a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity; and
(c) a sequence encoding a substance that inhibits cellular responsiveness to interferon.

117. The method of Claim 116, wherein the sequences of (a), (b) and (c) are DNA sequences and are stably expressed by the cell by integration into the cellular DNA or from an episome.

118. A helper cell for producing an alphavirus particle, comprising:
a cell having essentially no detectable interferon α/β (IFNAR) receptors, said cell, comprising:
(a) a sequence encoding an alphavirus structural protein; and

(b) a sequence encoding a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity.

119. The helper cell of Claim 118, wherein the sequences of (a) and (b) are DNA sequences and are stably expressed by the cell by integration into the cellular DNA or from an episome.

120. The helper cell of Claim 118, wherein said further has substantially reduced PKR activity.

121. The helper cell of any of Claims 116 to 120, wherein the cell further comprises a sequence(s) encoding the alphavirus structural proteins sufficient to produce an alphavirus particle.

122. The helper cell of any of Claims 116 to 121, wherein the cell further comprises an alphavirus replicon particle.

123. An isolated nucleic acid comprising (i) a sequence encoding at least one alphavirus structural protein, and (ii) a sequence encoding a substance that inhibits cellular responsiveness to interferon.

124. The isolated nucleic acid of Claim 123, further comprising (iii) a sequence encoding a substance that inhibits cellular double-stranded RNA-dependent (PKR) pathway activity in a cell.

125. A method of producing a recombinant alphavirus particle *in vitro*, comprising:

providing to a cell

(a) a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, said genomic RNA comprising a mutation at nucleotide 3 of the genomic RNA sequence and further comprising (i) a sequence(s) encoding the VEE nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence;

(b) a sequence(s) encoding the alphavirus proteins sufficient for producing an alphavirus particle; and

(c) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity;

wherein the combined expression of the recombinant VEE genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant VEE genomic RNA comprising the mutation at nucleotide 3 of the genomic RNA and the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

126. The method of Claim 125, wherein the method further comprises providing to the cell: (d) a substance that inhibits cellular responsiveness to interferon.

127. A method of producing a recombinant alphavirus particle, comprising:

providing to a cell having a reduced responsiveness to interferon, and optionally a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity:

(a) a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, said genomic RNA comprising a mutation at nucleotide 3 of the genomic RNA sequence and further comprising (i) a sequence(s) encoding the VEE nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence; and

(b) a sequence(s) encoding alphavirus structural proteins sufficient to produce an alphavirus particle;

wherein the combined expression of the recombinant VEE genomic RNA and the sequence(s) encoding the alphavirus structural proteins produces an assembled alphavirus particle comprising the recombinant VEE genomic RNA comprising the mutation at nucleotide 3 of the genomic RNA sequence and the heterologous nucleic acid sequence; and

producing an alphavirus particle in the cell.

128. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell

(a) a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, said genomic RNA comprising a mutation at nucleotide 3 of the genomic RNA sequence and further comprising (i) a sequence(s) encoding the alphavirus nonstructural proteins, (ii) a heterologous RNA sequence, and (iii) an alphavirus packaging sequence; and

(b) a substance that inhibits cellular double-stranded RNA-dependent protein kinase (PKR) pathway activity;

wherein the heterologous RNA sequence is introduced into and expressed in the cell.

129. The method of Claim 128, further comprising providing to the cell: (c) a substance that inhibits cellular responsiveness to interferon;

130. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell having a reduced responsiveness to interferon, and optionally a reduced double-stranded RNA-dependent protein kinase (PKR) pathway activity:

a recombinant Venezuelan Equine Encephalitis (VEE) genomic RNA, said genomic RNA comprising a mutation at nucleotide 3 of the genomic RNA sequence and further comprising (i) a sequence(s) encoding the VEE nonstructural proteins, (ii) a heterologous nucleic acid sequence, and (iii) an alphavirus packaging sequence;

wherein the heterologous RNA sequence is expressed in the cell.

5

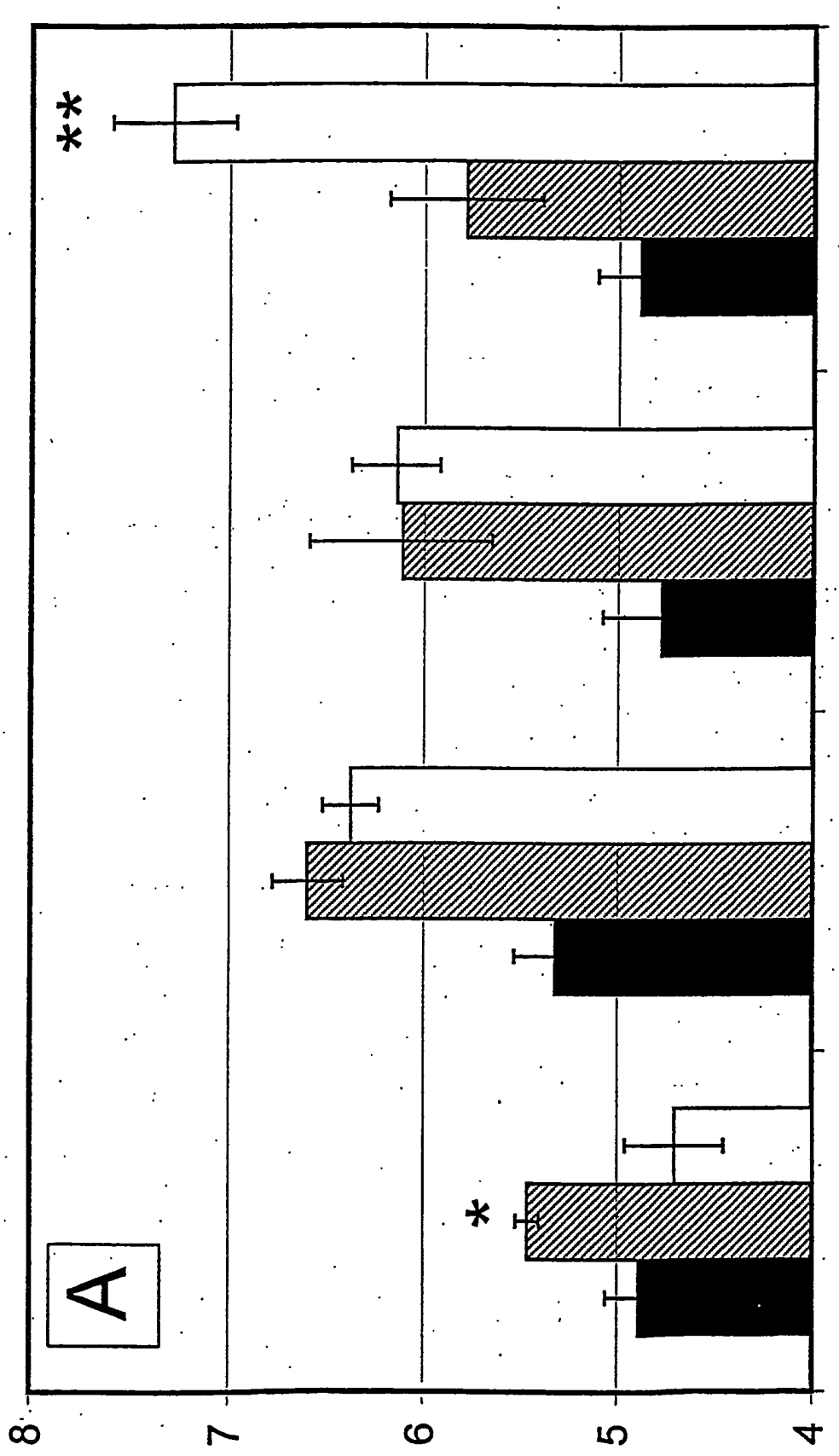
A
**
*
Geometric mean titer (Log10 PFU/g)
8
7
6
5
4
4
8
12
24
Hours post-infection

Fig 1 Panel A

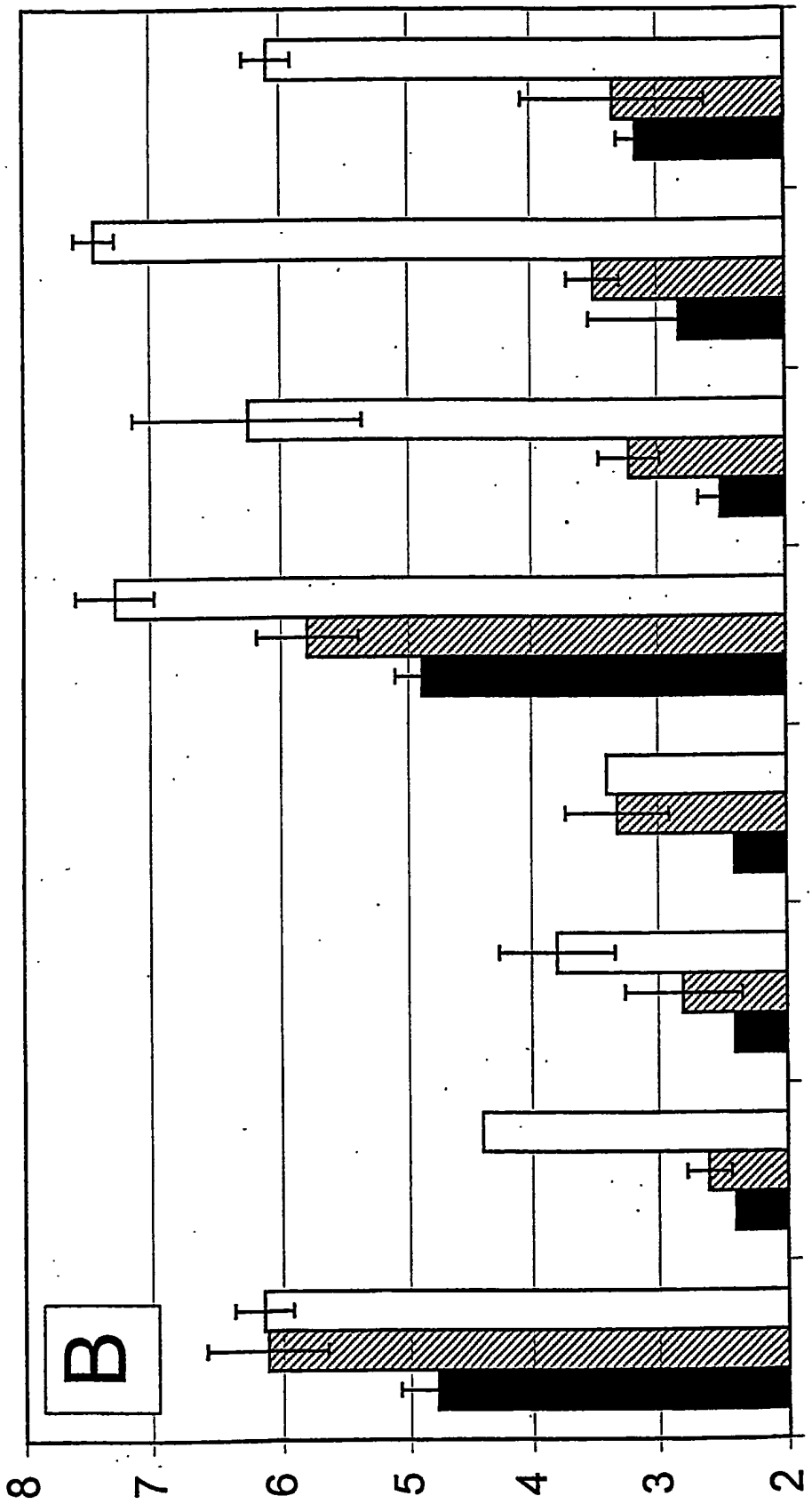
B
Geometric mean titer (Log10 PFU/ml or g)
8
7
6
5
4
3
2
LN
Serum
Spleen
Liver
12 h p.i.
LN
Serum
Spleen
Liver
24 h p.i.

Fig 1. Panel B

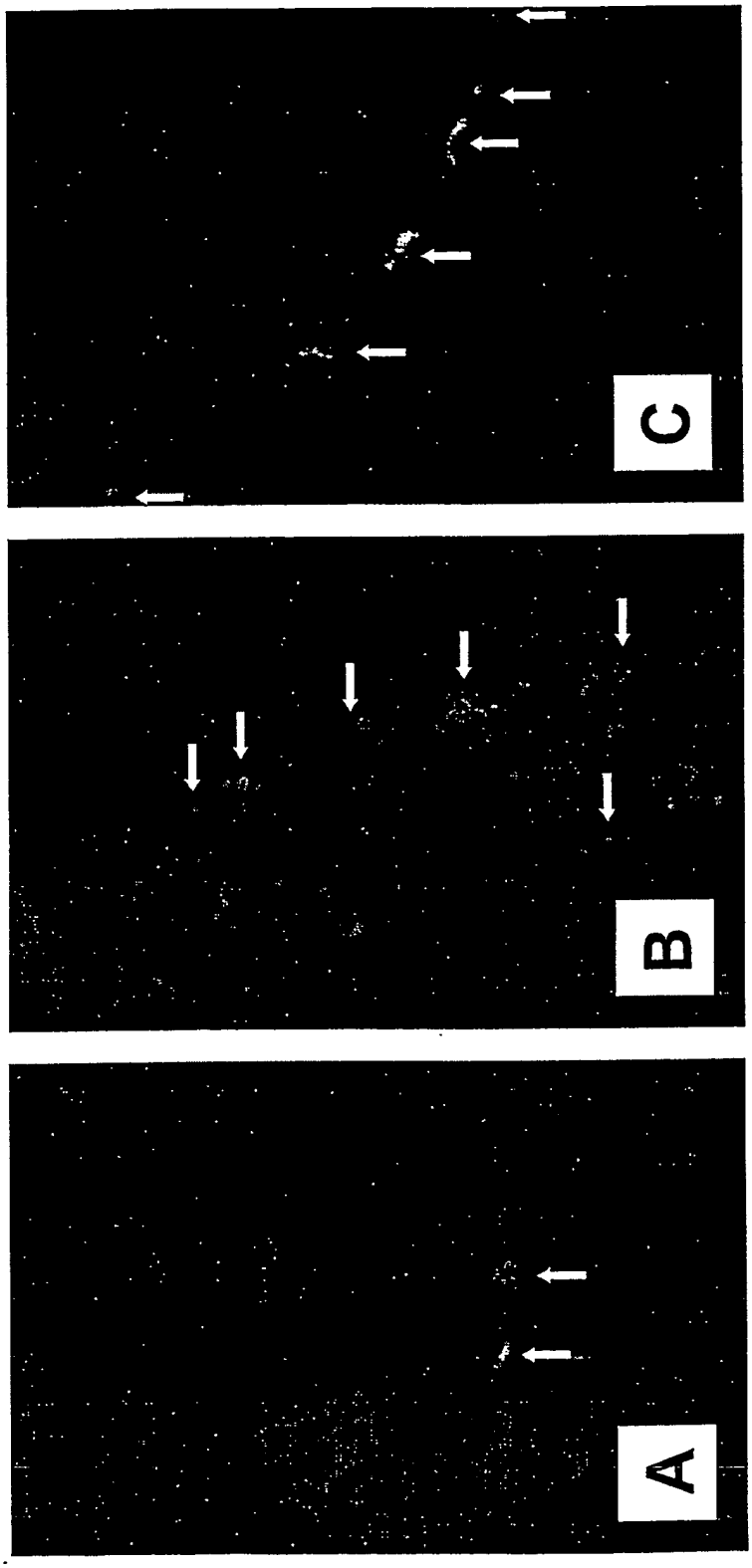

FIG 2

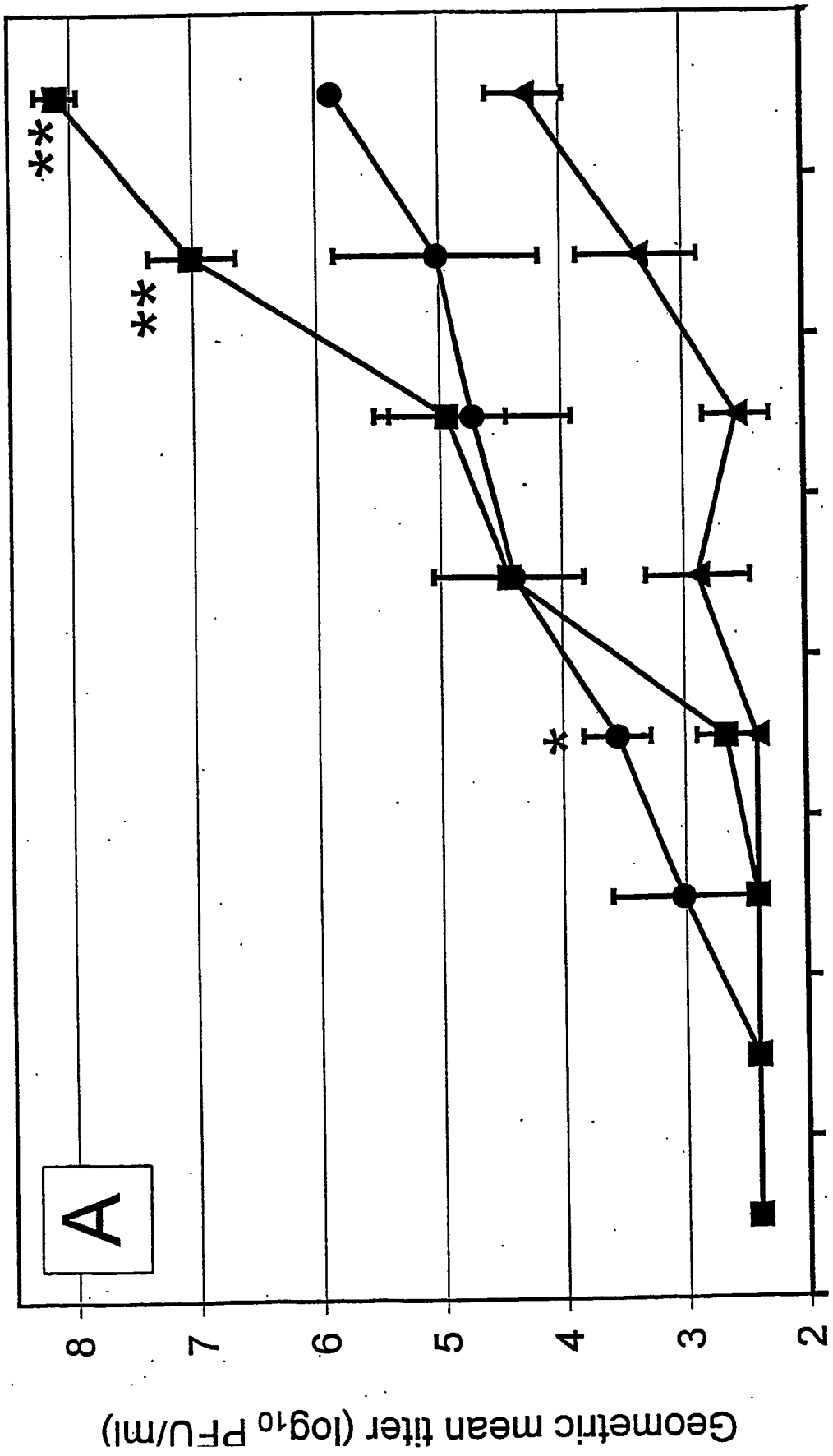
A
***
**
*
8
7
6
5
4
3
2
0
3
6
9
12
20
30
50
Hours post-infection
Geometric mean titer (log_{10} PFU/ml)

Fig. 3 Panel A

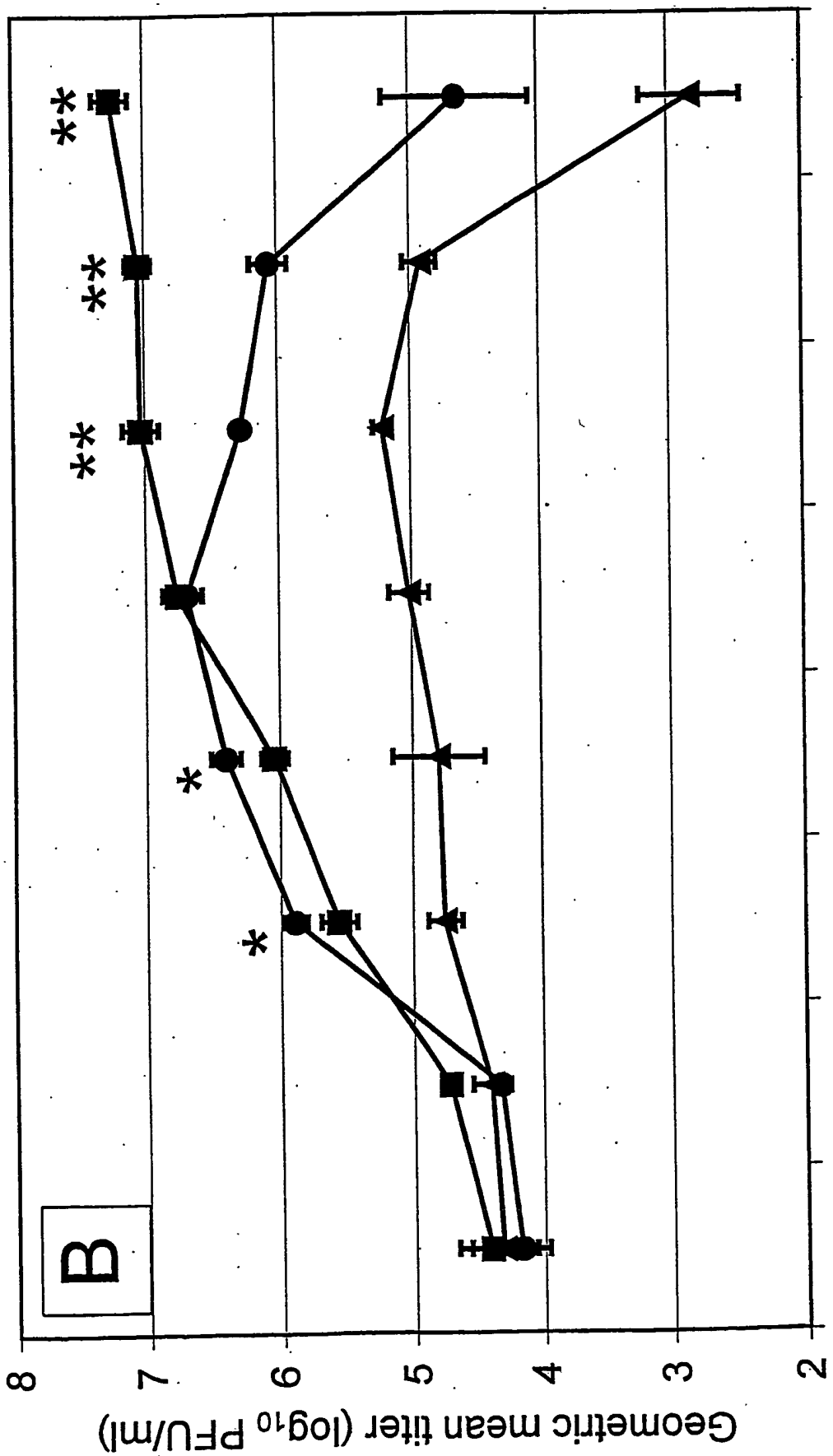
B
Geometric mean titer (log_{10} PFU/ml)
8
7
6
5
4
3
2
Hours post-infection
0
3
6
9
12
20
30
50
*
*
**
**
**

Fig 3 Panel B

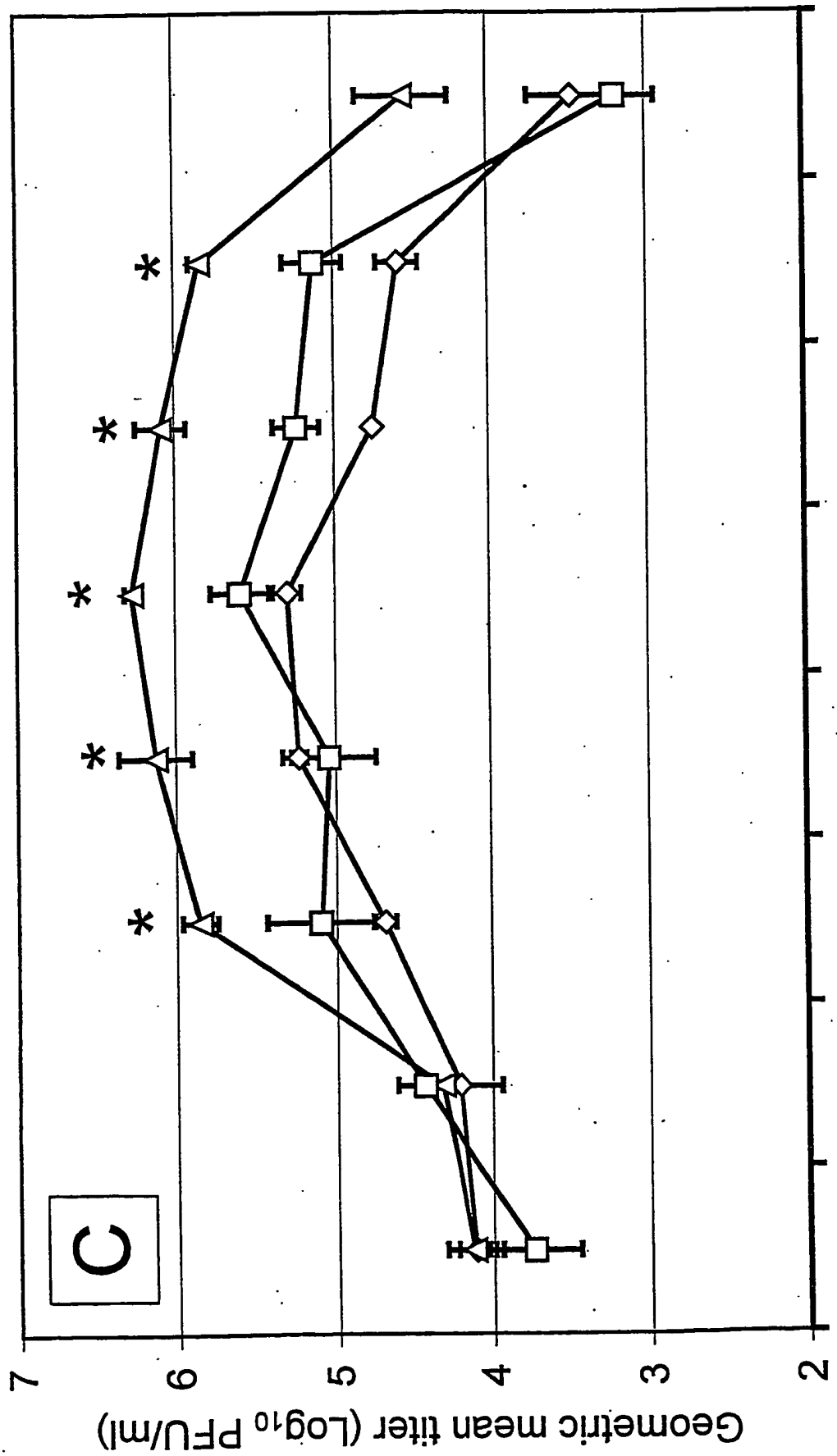
C
Geometric mean titer (Log10 PFU/ml)
7
6
5
4
3
2
0
3
6
9
12
20
30
50
Hours post-infection
*
*
*
*
*

Fig 3 Panel C

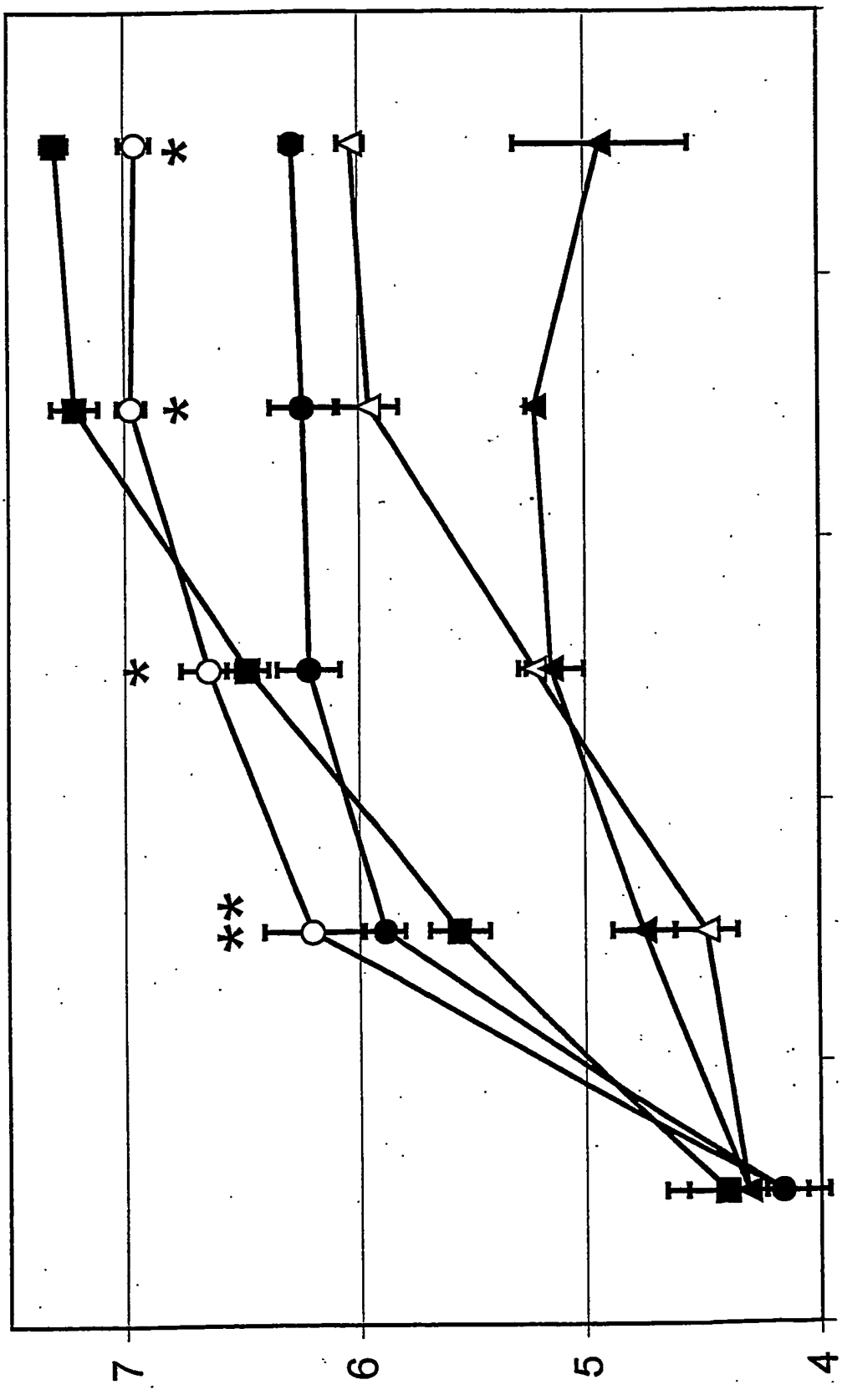
Geometric mean titer (log_{10} PFU/ml)
7
6
5
4
Hours post-infection
0
6
12
20
30
*
*
*
**

FIG 4

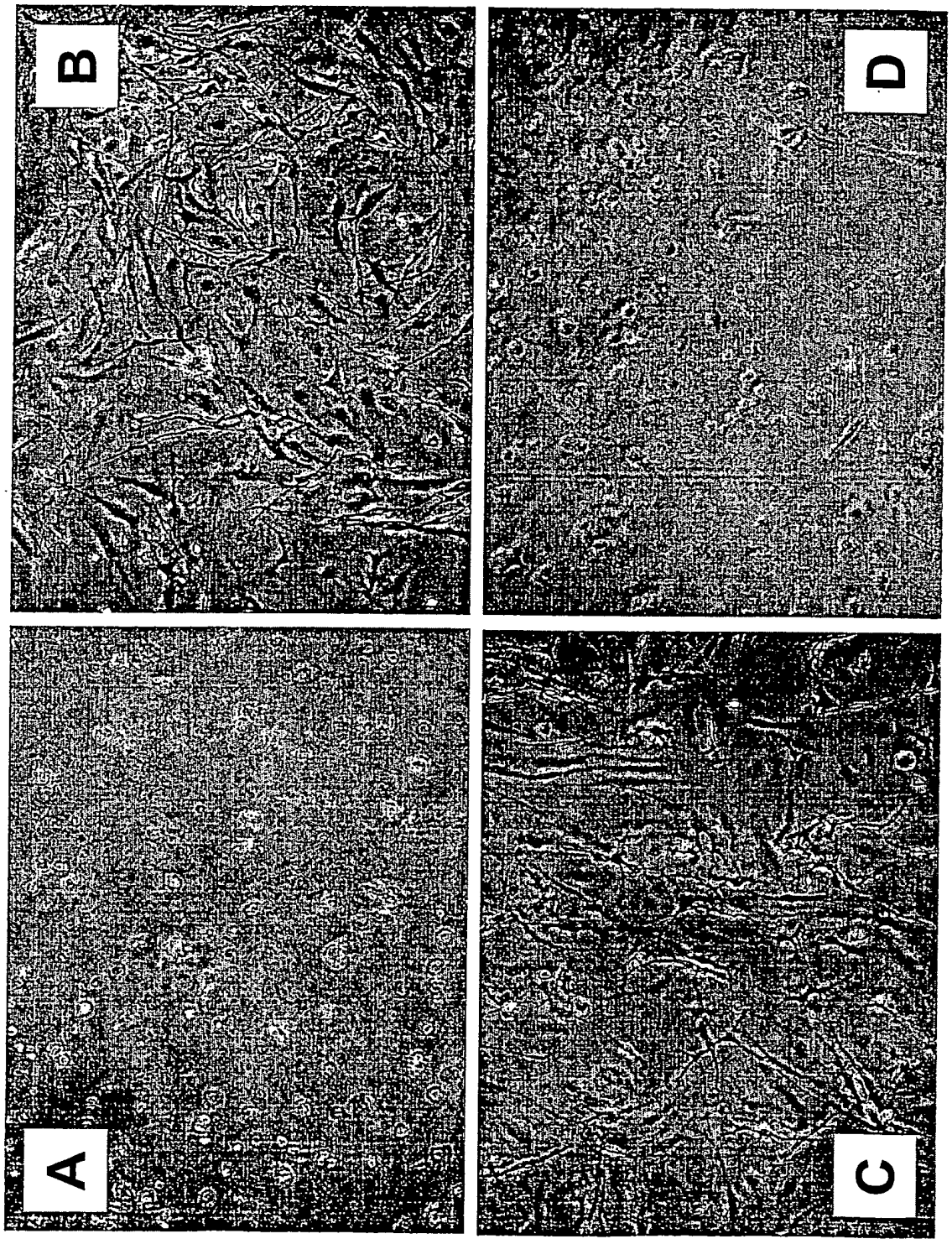

FIG 5

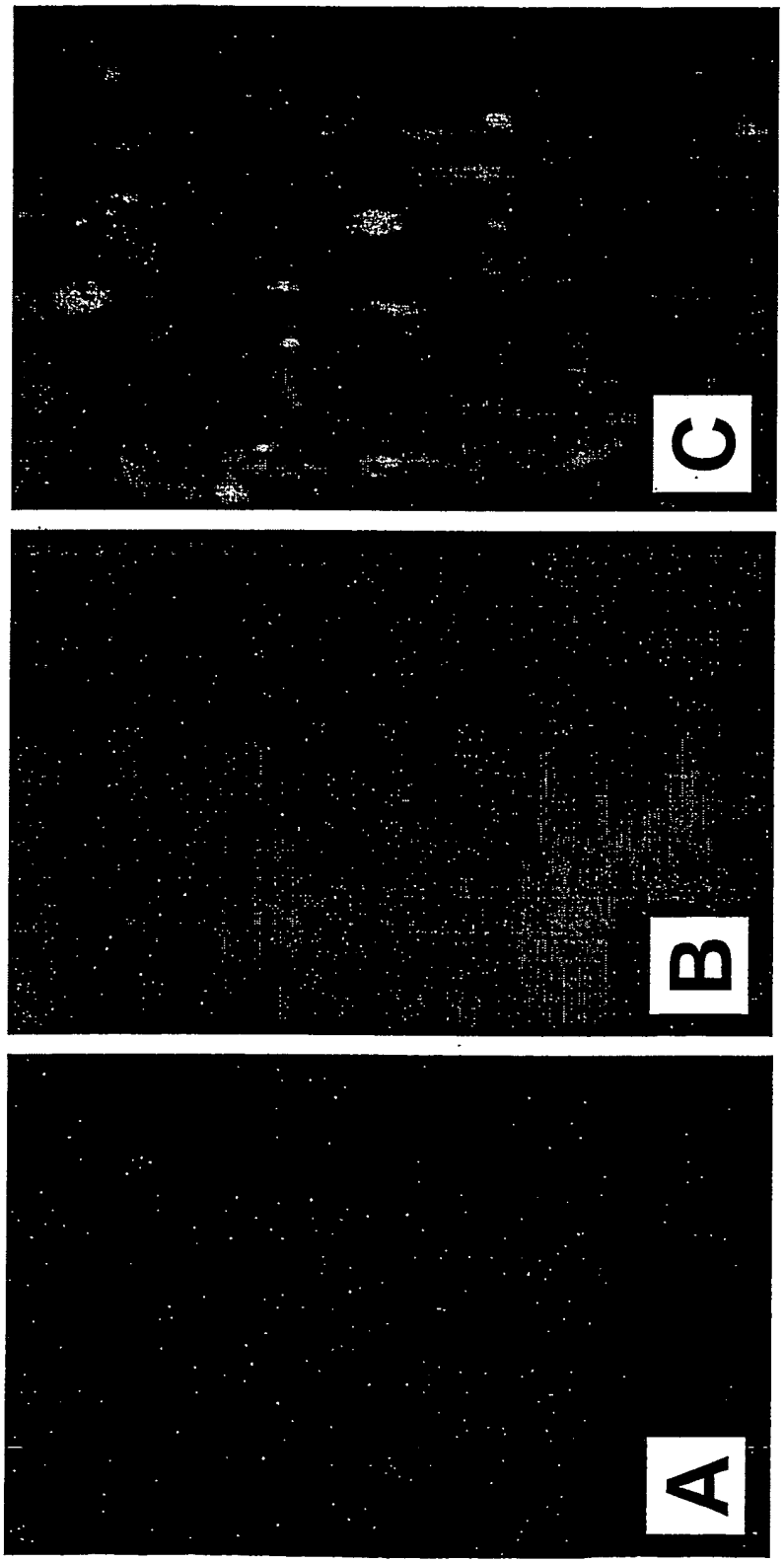

FIG6

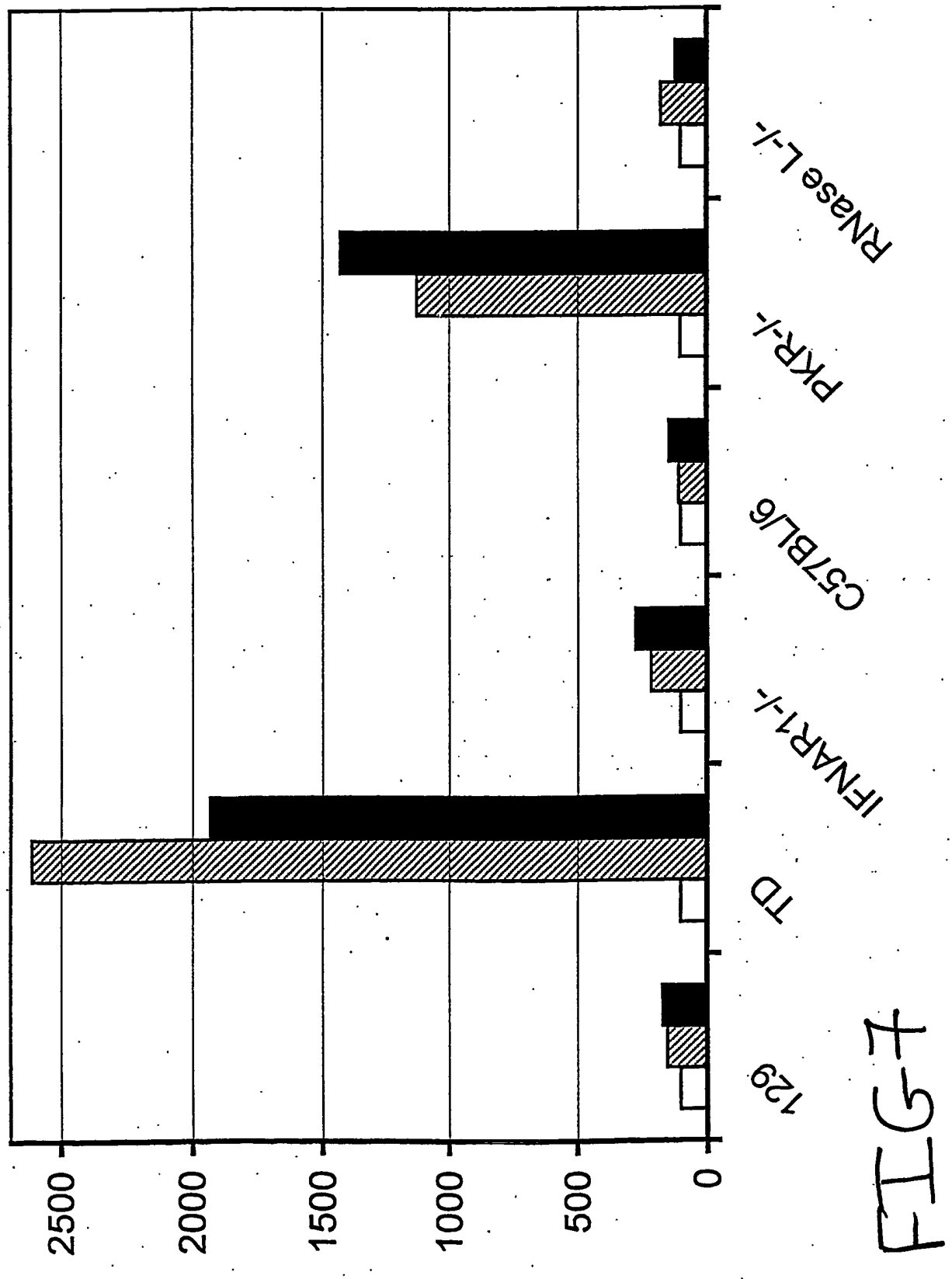
Mean Fluorescence Intensity
2500
2000
1500
1000
500
0
129
TD
IFNAR1-/-
C57BL/6
PKR-/-
RNase L-/-

FIG 7

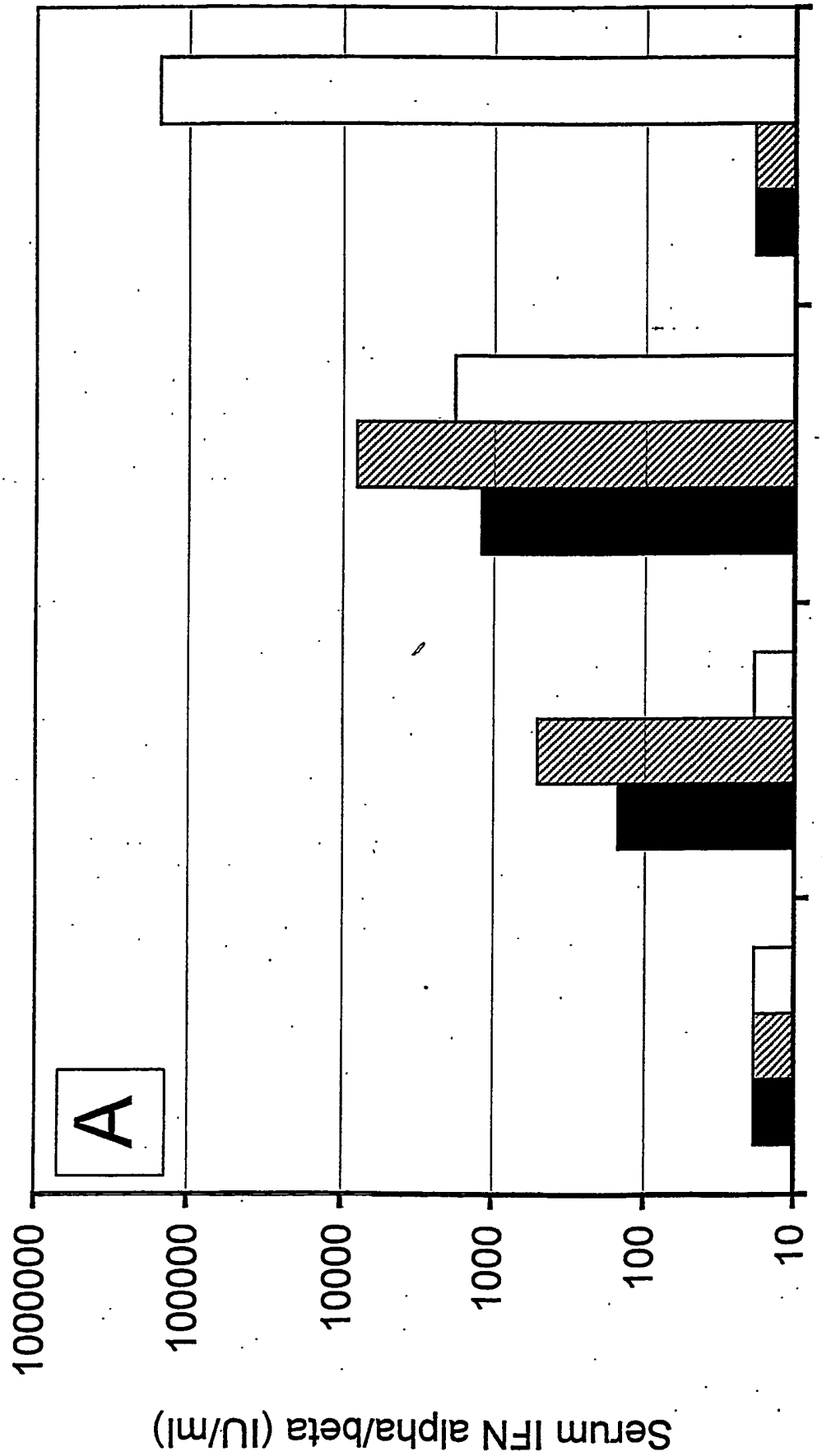
A
Serum IFN alpha/beta (IU/ml)
1000000
100000
10000
1000
100
10
4
8
12
24
Hours post-infection

FIG 8 PANEL A

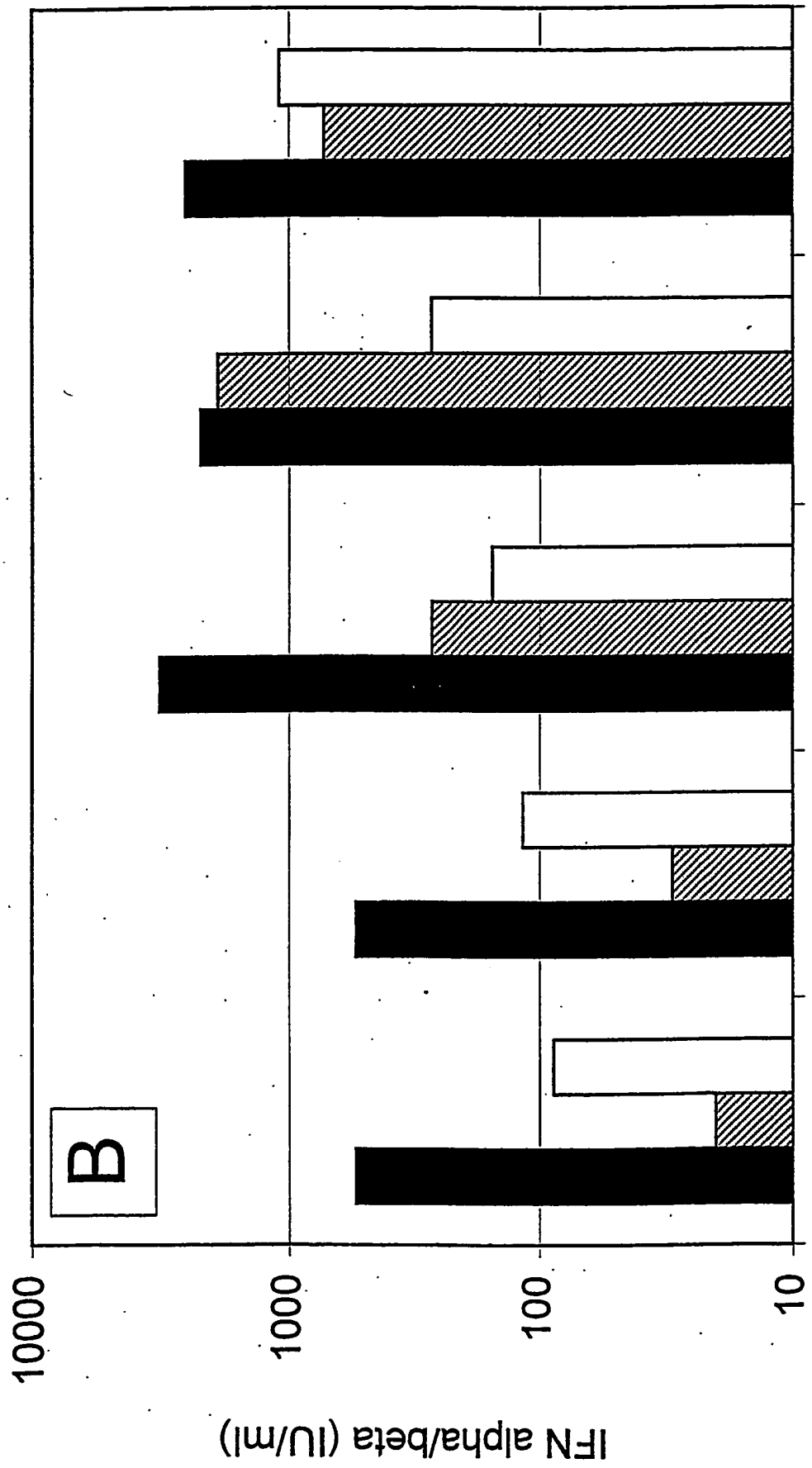
B
10000
1000
100
10
IFN alpha/beta (IU/ml)
3
6
9
12
20
Hours post-infection

FIG 8 PANEL B

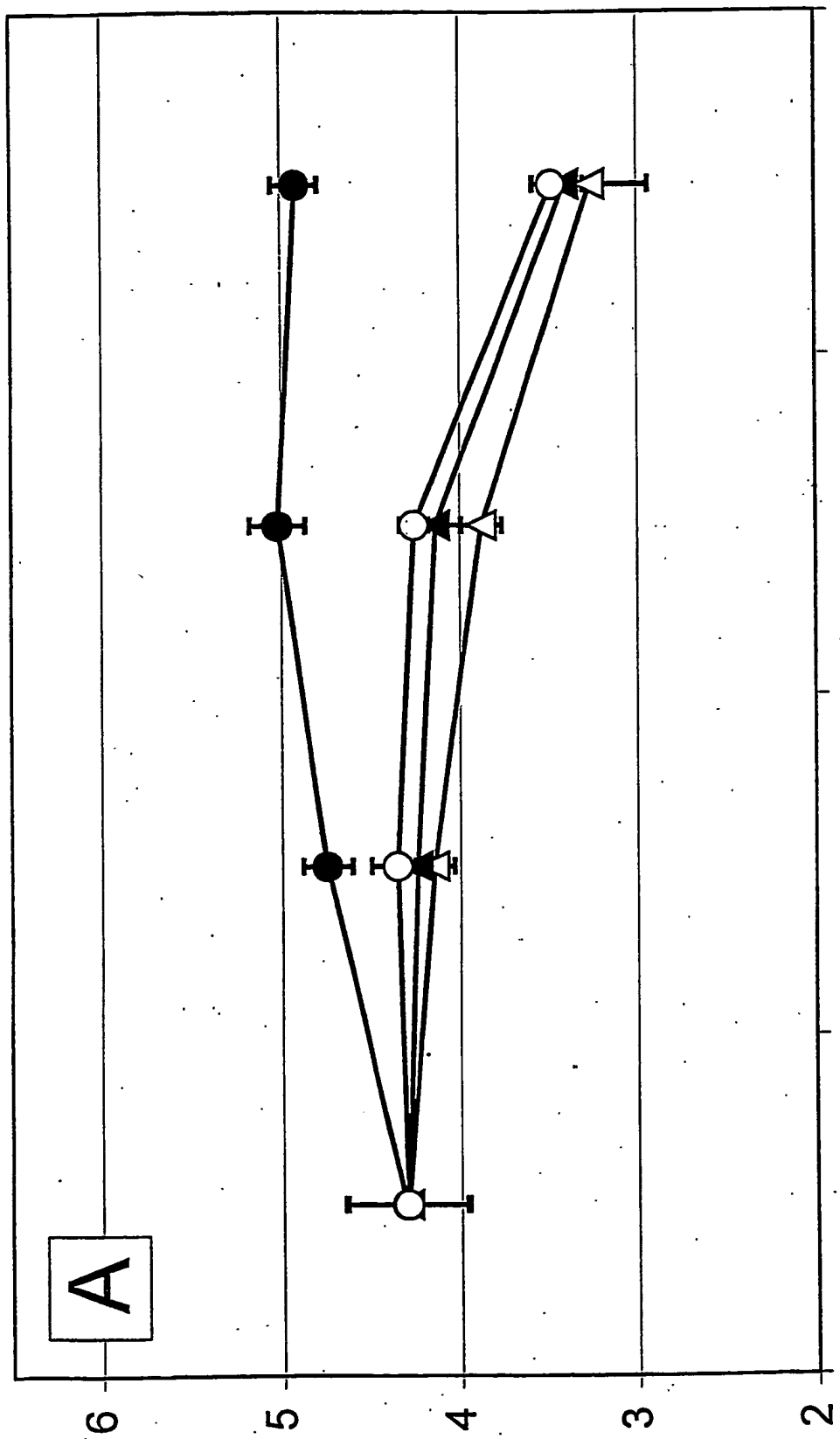
A
Geometric mean titer (Log10 PFU/ml)
6
5
4
3
2
0
6
12
30
Hours post-infection

FIG 9 PANEL A

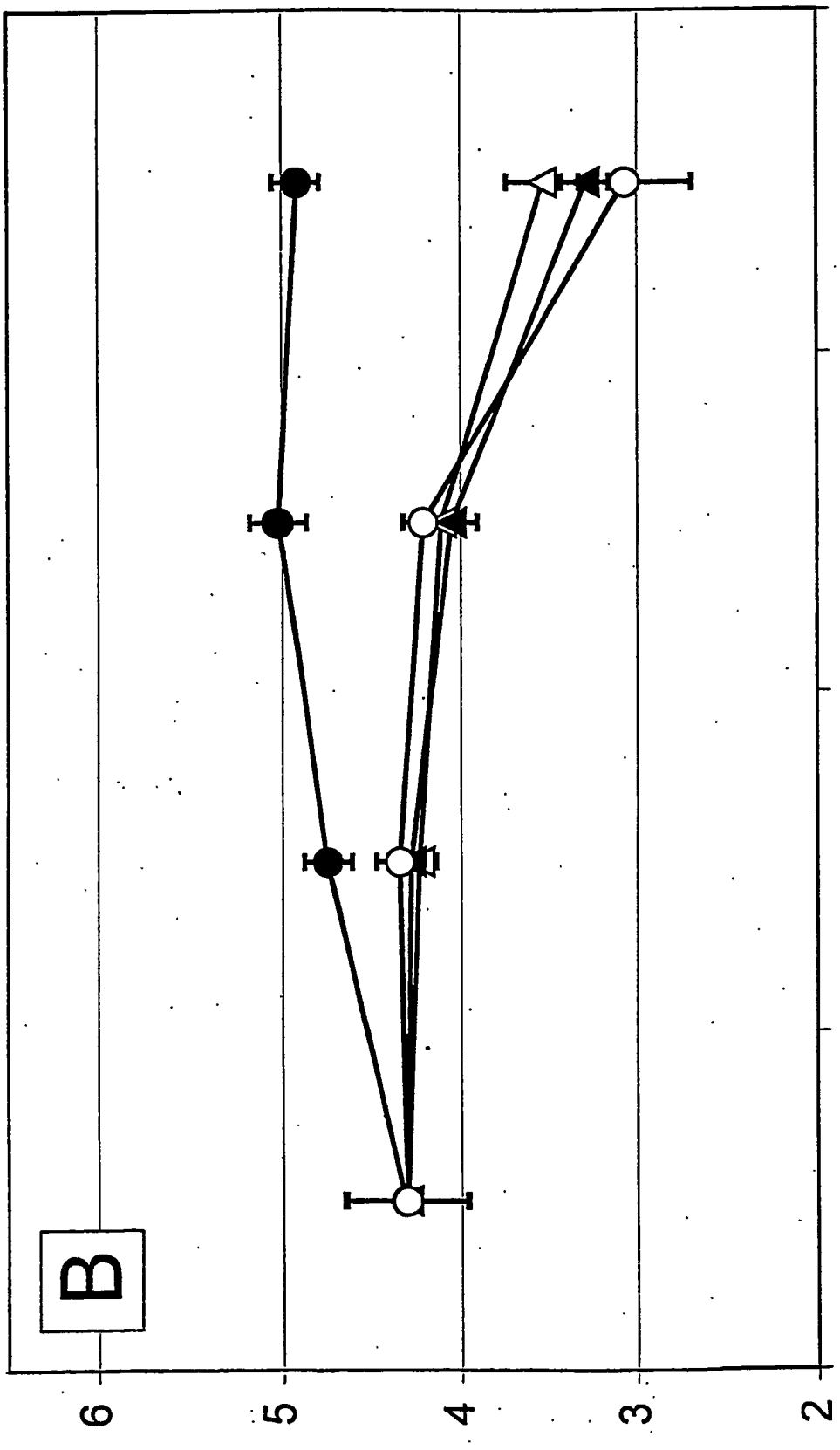
B
Geometric mean titer (Log10 PFU/ml)
6
5
4
3
2
0
6
12
30
Hours Post-infection

FIG 9 PANEL B

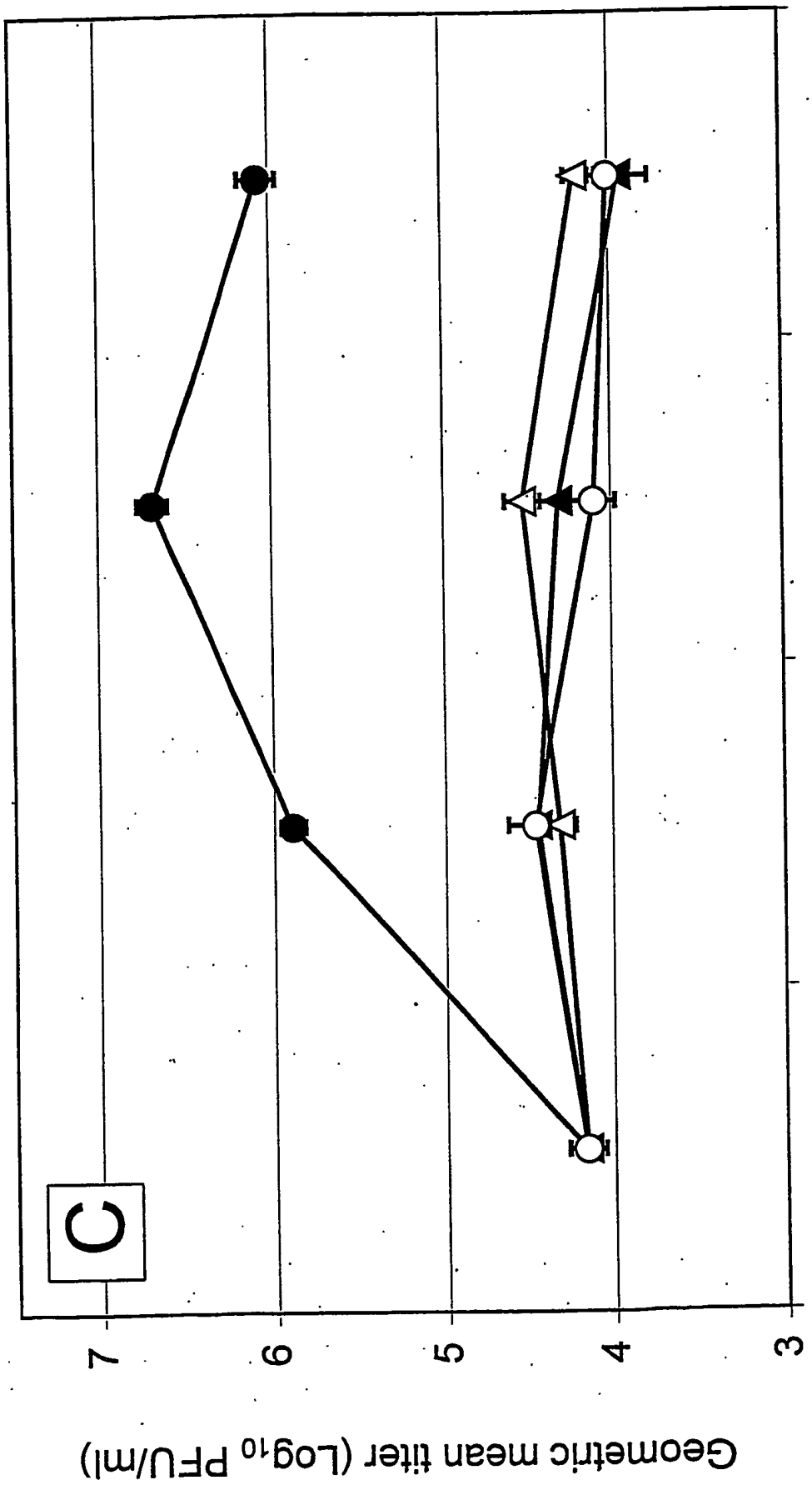
C
Geometric mean titer (Log10 PFU/ml)
7
6
5
4
3
0
6
12
30
Hours post-infection

FIG 9 PANEL C

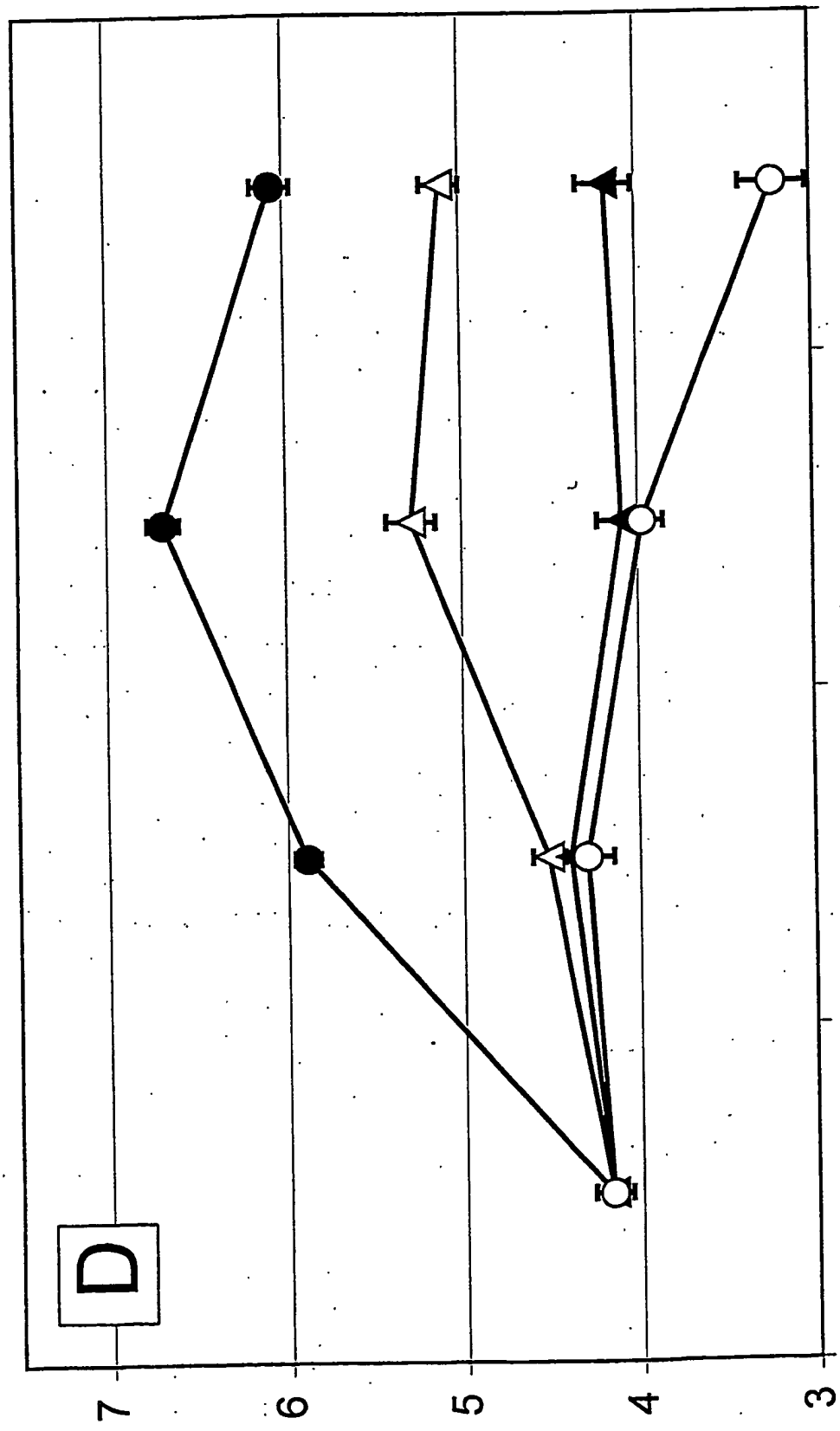
D
7
6
5
4
3
0
6
12
30
Hours post-infection
Geometric mean titer (Log10 PFU/ml)

FIG 9 PANEL D

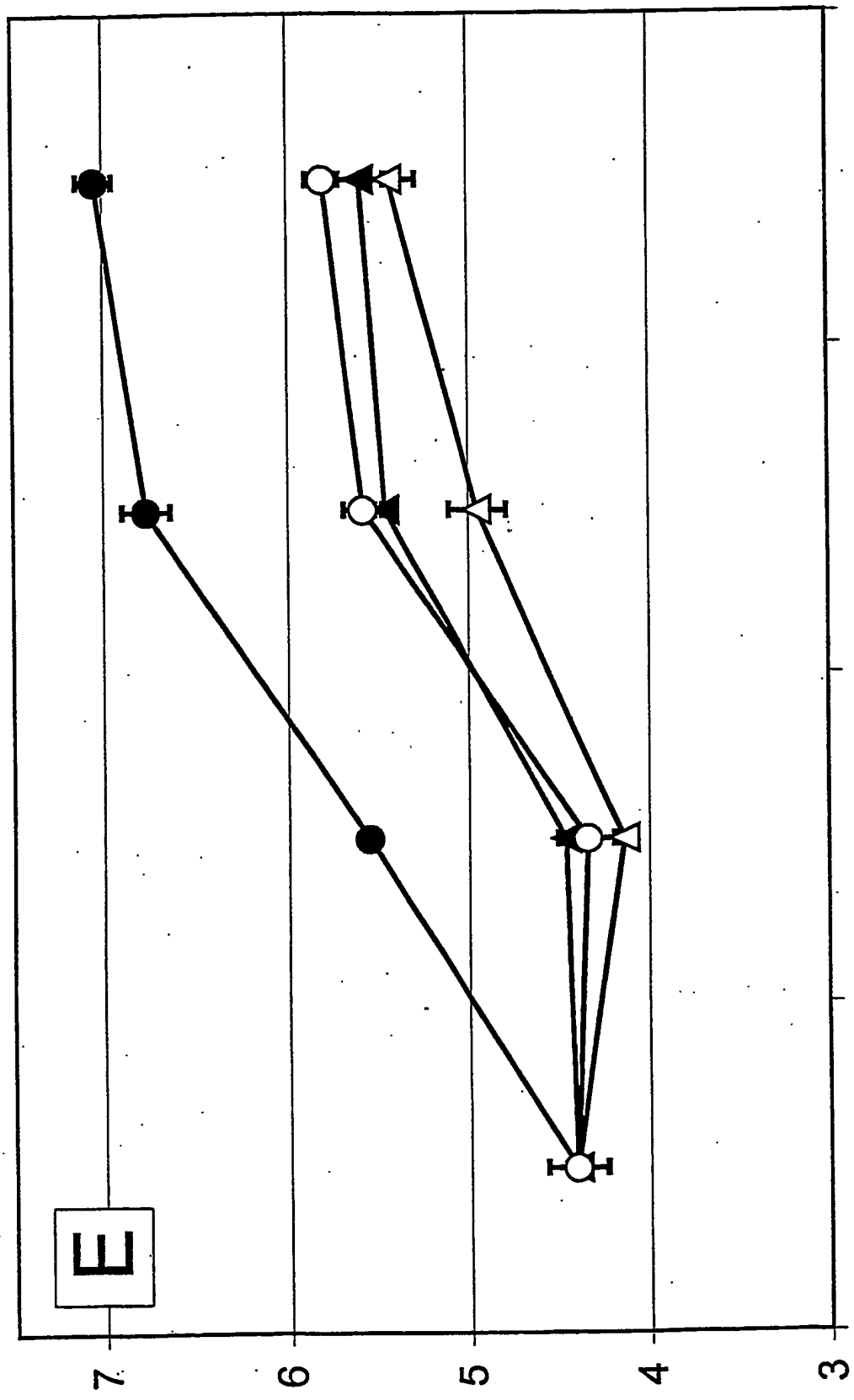
E
7
6
5
4
3
0
6
12
30
Hours post-infection
Geometric mean titer (Log_{10} PFU/ml)

FIG 9 PANEL E

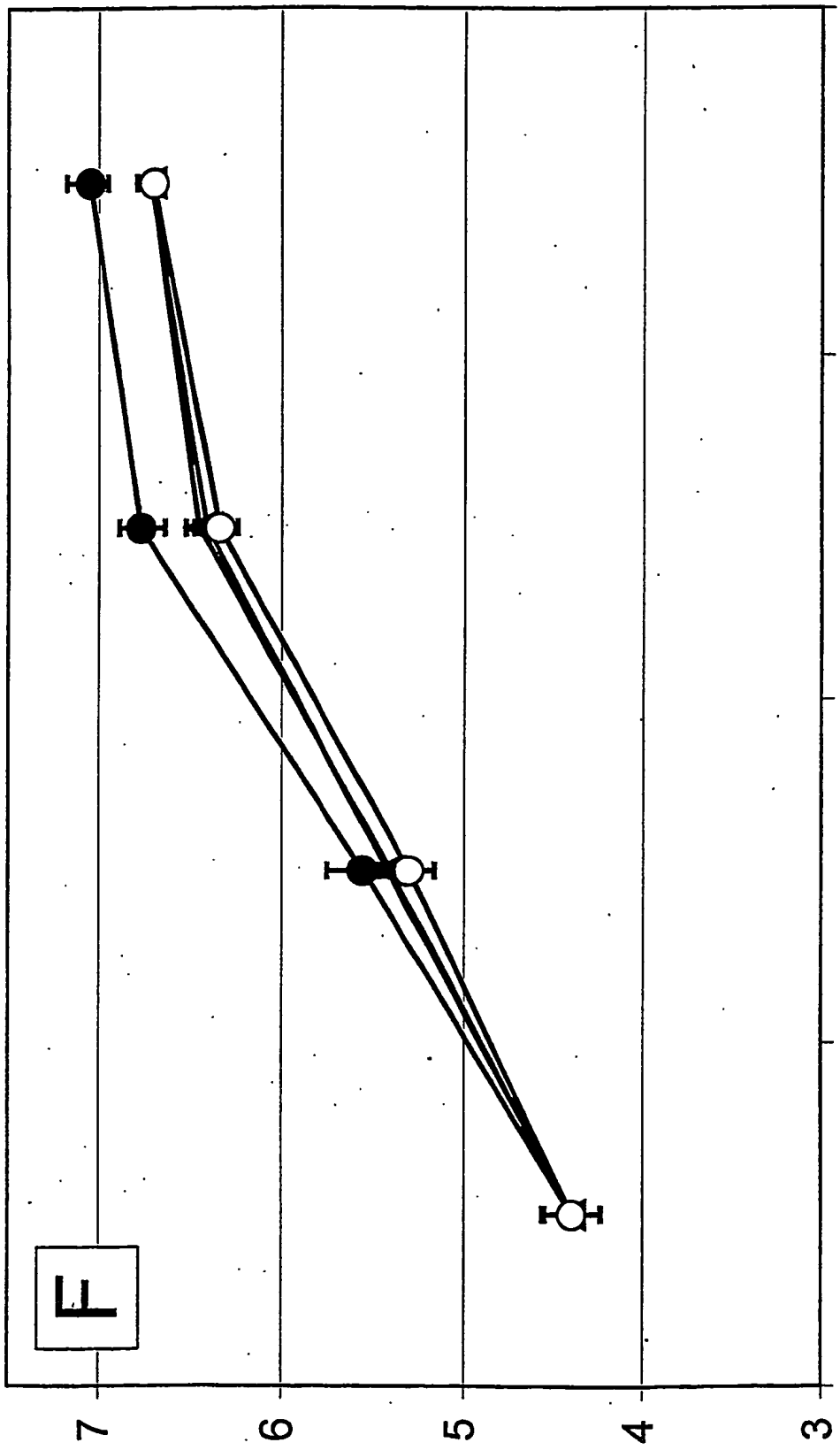
FL
Geometric mean titer (Log_{10} PFU/ml)
7
6
5
4
3
0
6
12
30
Hours post-infection

FIG 9 PANEL F

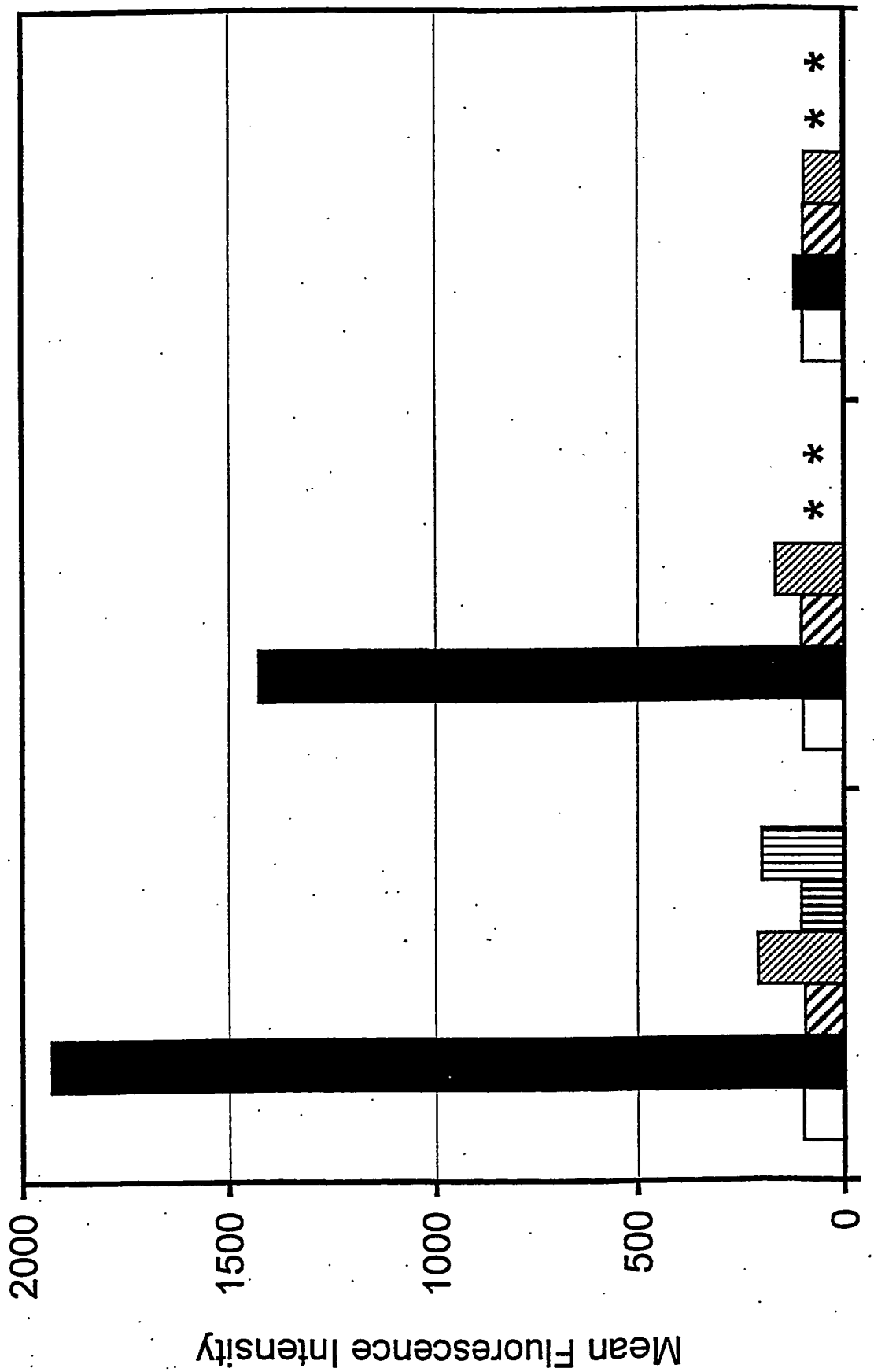
Mean Fluorescence Intensity
2000
1500
1000
500
0
TD
PKR-/-
RNase L-/-
* * * * * *

FIG 10